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Applicant: Christen M. Anderson, et al.

Application No.: 09/393,441

Filed: September 8, 1999

For: PRODUCTION OF ADENINE NUCLEOTIDE
TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR

Examiner : Sheridan Snedden
Art Unit : 1653
Docket No. : 660088.420C1
Date : November 3, 2003

DECLARATION Of CHRISTEN M. ANDERSON, M.D., PH.D.

UNDER 37 C.F.R. § 1.132

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I, Christen M. Anderson, a citizen of the United States, declare and state that:

1. I was a Scientist and Director of Metabolic Diseases at MitoKor, Inc., San Diego, California, United States, the assignee of the above-identified patent application (hereinafter referred to as "instant application"), at the time of filing, and I am a co-inventor on the instant application.

2. I have read the Office Action dated December 2, 2002, including the rejections of claims 42, 46-48, and 57 of the instant application under 35 U.S.C § 103.

3. Prior to the invention described in the instant application, there was a long-felt, unmet need in the art for recombinantly produced and isolated human ANT3 polypeptides and fusion proteins, particularly those that retained functional properties, including the ability to bind human ANT3 ligands. In addition, the successful production and isolation of recombinant human ANT3 polypeptides and fusion proteins had not been described at the time of filing the instant application, although numerous failed attempts had been made. My conclusions are based, in part, upon the evidence described below.

4. The skilled artisan would readily appreciate that there was a need in the art for recombinantly produced and isolated human ANT3 polypeptides and fusion proteins, given the fundamental role of ANT polypeptides in mitochondrial function, including human oxidative phosphorylation. It was known for some time that ANT polypeptides play a prominent role in respiratory, metabolic, apoptotic and other processes of the mitochondria, the altered function of which, in turn, is thought to be associated with numerous metabolic degenerative diseases. The skilled artisan would appreciate that in order to characterize the functional and structural properties of ANT3 polypeptides at the molecular level, to further understand their role in human disease, and potentially to identify therapeutic compounds suitable for treatment of mitochondrial-based disease, a reliable source of functional ANT3 polypeptides and fusion proteins is needed.

5. Furthermore, the skilled artisan would recognize that the need for recombinant human ANT3 polypeptides and fusion proteins was long-felt, since the important physiological role of ANT polypeptides was recognized as long ago as the early 1980s (e.g., Klingenberg, M. (1981) *Nature* 290, 449-454), yet recombinantly produced human ANT3 polypeptides and fusion proteins were not described before the instant application of 1998. Indeed, the attention directed to human and animal ANT polypeptides by numerous investigators, as evidenced by references cited throughout the instant application (see, e.g., page 15, lines 15-26; pages 39-40; Fiore *et al.* (1998) *Biochimie* 80, 137-150), and elsewhere, clearly demonstrates the recognition in the art of

a compelling need for a consistent, readily-produced and reliable source of ANT polypeptides.

6. Further, this long-felt, unmet need for recombinantly produced and isolated human ANT3 polypeptides and fusion proteins provides evidence of the failed attempts by others to produce these polypeptides. The cDNA sequence encoding human ANT3 was known as early as 1988 (Houldsworth, J. and Attardi, G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 377-381), and recombinant protein expression methods were known well before this date. Given the established need for recombinantly produced human ANT3 polypeptides and fusion proteins, the skilled artisan would immediately conclude that attempts to produce such polypeptides using known techniques had been made. It readily follows that the lack of any report or publication describing the successful production of recombinant human ANT3 polypeptides indicates that these attempts were unsuccessful.

7. In addition, direct evidence of failed attempts to produce and isolate recombinant human ANT polypeptides and fusion proteins is provided in numerous publications, including those described below.

(a) Fiermonte *et al.*, (1993) *Biochem. J.* 294, 293-299, describes failed efforts to express and reconstitute functional ANT polypeptides from bacteria. Fiermonte *et al.* illustrate some of the numerous difficulties associated with the expression and isolation of functional mammalian ANT polypeptides, including low yields due to mammalian ANT toxicity to host cells and the inability to isolate functional recombinant mammalian ANT polypeptides. Fiermonte *et al.* describe attempts to express and isolate two mitochondrial membrane transport proteins, the oxyglutarate carrier and a mammalian ANT polypeptide. Although Fiermonte *et al.* were able to express significant levels of the oxyglutarate carrier, they were only able to express low levels of non-functional ANT due to the toxicity of the ANT polypeptide in the bacterial cells. In particular, while Fiermonte *et al.* were able to isolate and reconstitute functional oxyglutamate, they were unable to isolate functional ANT polypeptides. This is indicated in their statement "[t]he expression of the ADP/ATP carrier in bacteria is also a step toward [an opportunity to study which amino acids are essential for the function of the carrier by

site-directed mutagenesis], although the essential reconstitution step has not yet been achieved." (emphasis added) (see Discussion, page 298). Clearly, this reference provides evidence of the failure of others to reconstitute a functional ANT polypeptide. Furthermore, this reference provides evidence of the difficulties specific to producing recombinant ANT polypeptides, as compared to other mitochondrial membrane transport proteins. Accordingly, this reference demonstrates unsuccessful attempts by others to produce isolated recombinant mammalian ANT polypeptides and further demonstrates that the production of recombinant ANT polypeptides was not routine, and that methods of doing so were not obvious to the skilled artisan.

(b) Further evidence of the failure of others to produce recombinant ANT protein is provided in Miroux *et al.* (1996) *Journal of Molecular Biology* 260(3), 289-298. Miroux *et al* describe multiple problems with regard to efforts to express recombinant ANT, including toxicity to host cells, poor solubility of the recombinant product, and the accumulation of recombinant ANT in inclusion bodies. The skilled artisan would appreciate the persistent and challenging problems associated with isolating functional polypeptides from inclusion bodies. Extracting insoluble protein under non-denaturing conditions using detergents and sonication is time consuming and yields low levels of protein, and cannot always be expected to result in a functional protein. Extracting a protein under denaturing conditions with urea or other reagents is often successful, but using these methods requires refolding the protein and frequently results in a loss of structure and activity. Based upon this understanding, I submit that the isolation of functional ANT polypeptides from inclusion bodies is not routine, and the skilled artisan would have no reasonable expectation of being able to successfully isolate functional ANT polypeptides based upon Miroux *et al.*'s teaching that ANT polypeptides accumulate in inclusion bodies.

(c) Rojo and Wallimann ((1994) *Biochim et Biophysica Acta* 1187, 360-367) describe efforts to purify ANT polypeptides from tissue using various detergents, and demonstrate the difficulties associated with purifying and reconstituting functional ANT polypeptides. Rojo and Walliman note that "[a]lthough various detergents have been the subject of systematic studies concerning multiple aspects of

their interaction with membranes and membrane proteins, still no rationale exists that allows us to identify *a priori* the detergent or detergent class that is suited for a particular protein and/or application. Thus, detergents have to be selected based upon the basis of empirical studies." This reference indicates that methods successful in the purification or isolation of ANT polypeptides are unpredictable, and the skilled artisan would, given the state of the art at the time of filing the present application, have had no reasonable expectation of successfully isolating functional ANT polypeptides using any particular method. Furthermore, this reference provides additional evidence establishing the unpredictability in isolating functional polypeptides from inclusion bodies.

8. In conclusion, I submit that the above-cited references provide evidence of a long-felt need for and the failure of others to successfully express, purify and reconstitute recombinant ANT polypeptides and fusion proteins. In addition, I submit that these references clearly indicate that the production of functional ANT polypeptides and fusion proteins is not routine and that without the teaching of the present application, the skilled artisan would have no reasonable expectation of successfully producing and isolating ANT polypeptides.

9. The undersigned declares further that all statements made herein of her own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false statements, and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.



Christen M. Anderson, M.D., Ph.D.

11/10/03
Date



COMMUNICATION

Over-production of Proteins in *Escherichia coli*: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels

Bruno Miroux and John E. Walker*

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The Medical Research
Council Laboratory of
Molecular Biology
Hills Road, Cambridge
CB2 2QH, UK

We have investigated the over-production of seven membrane proteins in an *Escherichia coli*-bacteriophage T7 RNA polymerase expression system. In all seven cases, when expression of the target membrane protein was induced, most of the BL21(DE3) host cells died. Similar effects were also observed with expression vectors for ten globular proteins. Therefore, protein over-production in this expression system is either limited or prevented by bacterial cell death. From the few survivors of BL21(DE3) expressing the oxoglutarate-malate carrier protein from mitochondrial membranes, a mutant host C41(DE3) was selected that grew to high saturation cell density, and produced the protein as inclusion bodies at an elevated level without toxic effect. Some proteins that were expressed poorly in BL21(DE3), and others where the toxicity of the expression plasmids prevented transformation into this host, were also over-produced successfully in C41(DE3). The examples include globular proteins as well as membrane proteins, and therefore, strain C41(DE3) is generally superior to BL21(DE3) as a host for protein over-expression. However, the toxicity of over-expression of some of the membrane proteins persisted partially in strain C41(DE3). Therefore, a double mutant host C43(DE3) was selected from C41(DE3) cells containing the expression plasmid for subunit b of bacterial F-ATPase. In strain C43(DE3), both subunits b and c of the F-ATPase, an alanine-H⁺ symporter, and the ADP/ATP and the phosphate carriers from mitochondria were all over-produced. The transcription of the gene for the OSCP and subunit b was lower in C41(DE3) and C43(DE3), respectively, than in BL21(DE3). In C43(DE3), the onset of transcription of the gene for subunit b was delayed after induction, and the over-produced protein was incorporated into the membrane. The procedure used for selection of C41(DE3) and C43(DE3) could be employed to tailor expression hosts in order to overcome other toxic effects associated with over-expression.

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*Corresponding author

Keywords: *E. coli*; T7 RNA polymerase; over-expression; membrane proteins

Abbreviations used: OSCP, oxoglutarate-malate transport protein from mitochondria; F-ATPase, H⁺-transporting F_F-ATPase; OSCP, oligomycin sensitivity conferral protein, a subunit of bovine F-ATPase; GFP, green fluorescent protein from the jelly-fish, *Aequoria victoria*; IPTG, isopropyl-2-D-thio-galactopyranoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; expression plasmid names consist of the name of the plasmid vector, followed (in parentheses) by the recombinant they encode.

Escherichia coli is one of the most successful vehicles for over-expression of both prokaryotic and eukaryotic proteins (for reviews see Hockney, 1994; Grisshammer & Tate, 1995). However, the

† Here, the term "over-expression" implies that the target protein is expressed in the *E. coli* cells at a level that would provide a convenient source of material for structural studies. Minimally this is likely to be about 1 mg of the target protein per litre of bacterial culture.

expression vectors for many membrane proteins, as well as for some cytoplasmic proteins (Dong *et al.*, 1995), for cell division proteins (de Boer *et al.*, 1988; Gutzman *et al.*, 1992) and for other toxic proteins such as DNase (Doherty *et al.*, 1993) kill the host bacterium. Therefore, we have investigated the toxicity of over-expression of membrane proteins in *E. coli* in one of the most widely used expression systems, in which the target gene is transcribed from the vector by bacteriophage T7 RNA polymerase (Studier *et al.*, 1990). In the *E. coli* BL21(DE3) host strain, used in conjunction with pET vectors, the T7 RNA polymerase is produced from the λ -lysogen DE3 in the host bacterium, and its expression is under the control of the IPTG-inducible lac UV5 promoter. This system has been employed successfully for over-production of many globular proteins, but in many other cases significant over-production cannot be achieved because of the toxicity of over-expression (Studier *et al.*, 1990; George *et al.*, 1994).

The toxic effects of the over-expression of seven membrane proteins (see Table 1) cloned in pET and

related expression plasmids towards the *E. coli* BL21(DE3) host cells were investigated by attempting to grow cells containing the plasmids on two sets of agar plates, one containing IPTG and the other lacking the inducer. The proteins investigated were the OSCP, the phosphate carrier and the ADP/ATP carrier, all three being members of a super-family of transport proteins with six transmembrane spans (Walker & Runswick, 1993), subunits b and c of the *E. coli* F-ATPase with one and two transmembrane spans, respectively (Fillingame, 1990), and a fusion protein between bacteriophage T7 10a protein and the alanine-H⁺ carrier from *Bacillus* PS3, which is thought to have 10 to 12 transmembrane α -helices (Kamata *et al.*, 1992). In earlier studies, the OSCP had been shown to be over-produced at high levels (10 mg per litre of bacterial culture) in *E. coli* BL21(DE3) (Fiermonte *et al.*, 1993). None of the seven vectors produced colonies on the plates containing IPTG, and in the absence of IPTG only very small colonies formed from cells containing plasmids for the b-subunit of *E. coli* F-ATPase, for the bovine ADP/ATP carrier,

Table 1. Expression of various proteins in BL21(DE3), C41(DE3) and C43(DE3) hosts

Protein*	Location*	Expression level†		
		BL21	C41	C43
Bovine OSCP (m)	IB	10 ¹	100 ¹	84 ¹
Bovine phosphate carrier (m)	IB	5 ¹	35 ¹	52 ¹
<i>Bacillus</i> PS3 alanine/H ⁺ carrier (m)	IB	—	9 ¹	18 ¹
<i>E. coli</i> F-ATPase subunit b (m)	IB	—	19 ²	79 ²
<i>E. coli</i> F-ATPase subunit c (m)	IB/M	—	8 ²	25 ²
Bovine F-ATPase subunit b (m)	M	2 ²	10 ²	15 ²
Bovine F-ATPase subunit a (g)	IB	ND	30 ²³	ND
Bovine F-ATPase subunit β (g)	IB	35 ²	135 ²	ND
Bovine F-ATPase subunit γ (g)	IB	50 ²	240 ²	ND
Bovine F-ATPase subunit δ (g)	IB	11 ²	74 ²	ND
Bovine F-ATPase subunit d (g)	IB	4 ²	18 ²	ND
Bovine F-ATPase subunit OSCP (g)	IB	10 ²	20 ²	3 ²
Bovine F-ATPase subunit P _c (g)	IB	50 ¹	300 ¹	ND
Bovine F-ATPase inhibitor protein (g)	C	65 ²	130 ²	ND
<i>D. melanogaster</i> staufen protein (g)	C	8 ²	70 ²	ND
<i>Aequorea victoria</i> GFP (g)	IB/C	—	ND ⁴	ND
		37 ²	140 ²	ND

At the end of the expression experiment (three hours growth in BL21), 18 hours in C41 in most cases), cells were centrifuged (7000 g, 10 minutes) and re-suspended in buffer (20 ml) consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 0.001% (w/v) phenylmethylsulphonyl fluoride. Bacteria containing over-produced proteins were passed twice through a French pressure cell (pre-cooled to 4°C), inclusion bodies were collected by centrifugation (10 minutes, 10,000 g), and the membrane and cytosolic fractions were separated by ultra-centrifugation (two hours, 100,000 g).

* m, membrane protein, g, globular protein.

† IB, inclusion bodies; C, soluble in cytosol; M, in membrane; for *E. coli* F-ATPase subunit b, IB/M indicates that, C41(DE3), the protein was in a form that was difficult to solubilize in detergent, but in C43(DE3) it was in the membrane and was readily detergent extractable (see the text); for the GFP, IB/C indicates that the protein was partially soluble and partially in inclusion bodies in both BL21(DE3) and C41(DE3).

The expression level is given as mg protein/litre of bacterial cells, quantified by: 'bicinchoninic acid assay, or ³⁵N-terminal sequencing. A portion of cells was solubilized in 1% SDS, and the proteins were separated by SDS-PAGE (Laemmli, 1970), transferred to polyvinylidene difluoride membranes and stained with PAGE 83 dye. Appropriate bands were excised and introduced into the sequencer. The yields of phenylthiohydantoin released at each of 15 consecutive cycles of Edman degradation were measured by HPLC, and the amount of the protein of interest on the membrane was estimated by extrapolation to cycle zero. Each experiment was performed twice. The bovine F-ATPase b-subunit probably has two trans-membrane spanning α -helices and is not related in sequence to the *E. coli* b-subunit, which has one trans-membrane span (Walker *et al.*, 1987); the staufen protein (St Johnston *et al.*, 1991) was detected in the soluble fraction of the cells by Western blotting by D. St Johnston. A hyphen indicates that because of toxicity of the expression plasmid, no expression was obtained. ND, not determined. With the exceptions of the alanine-H⁺ carrier, which was cloned in pCGT180 (kindly donated by Dr C. G. Tate, it was derived from pGEMX and produces a fusion protein with the major capsid protein 10A of phage T7), and the staufen protein, which was cloned into pET7, the coding sequences of the various proteins were cloned into pMW7 (Way *et al.*, 1990). Also, see Collinson *et al.* (1994) and Orriss *et al.* (1996) for more details of vectors, and Chalfie *et al.* (1994) for details of the GFP. The GFP was cloned from a mutant, GFPA (K. Siemerling, R. Goblik & J. Haseloff, unpublished results).

and for the alanine-H⁺ carrier. The cells in these very small colonies were not viable, and therefore it would not be possible to produce inocula for over-expression cultures in liquid media. Small viable colonies were obtained with the plasmid for *E. coli* subunit c. Similar experiments were conducted with vectors for ten globular proteins (see Table 1); none of them formed colonies in the presence of IPTG. Therefore, all 17 of the expression plasmids that were examined produced toxic effects on the BL21(DE3) host, with a wide spectrum of severity. The plasmids encoding membrane proteins were the most toxic, but among plasmids encoding membrane proteins, the one encoding the OGCP was the least toxic.

A control experiment was conducted with five different expression vectors from the pET family, all of them lacking a target gene for possible over-expression. They were pMW7, pET17b (containing an N-terminal T7 tag), pET23a (containing a N-terminal T7 tag and a C-terminal His-tag), pET29a (containing an S-tag) and pGEMEX-1 (containing a gene 10a fragment). Surprisingly, none of the cells containing the "empty" plasmids produced colonies in the presence of IPTG, except for pET 17b, which gave very small colonies, demonstrating that the plasmids themselves are intrinsically toxic to *E. coli* BL21(DE3) host cells. Therefore, the toxicities for expression plasmids containing target genes, consist of contributions from both the vector (and associated tags) and the target gene.

The inhibitory effects of the pMW7 (OGCP) expression vector on *E. coli* BL21(DE3) were also studied in liquid culture containing ampicillin. The culture was grown for four hours before addition of the inducer, IPTG (see phase 1 in Figure 1A). One hour or so later, the cells had stopped dividing and the absorbance of the culture decreased (phase 2). After a further five to six hours, the absorbance rose again (phase 3) and eventually reached a value greater than 5. The maximal level of expression of the OGCP was attained three hours after addition of inducer, and it diminished thereafter. Therefore, phase 3 corresponds to the outgrowth of cells that had lost the ability to express the target gene. Similar three-phase growth curves have been observed in our studies of all of the proteins that have been over-produced in *E. coli* BL21(DE3). Some variation was observed in the length of the lag in phase 1 from three hours (GFP) to ten hours (*E. coli* F-ATPase subunit c), and in maximal cell density in phase 2 from 0.5 to 2.0, depending on the degree of toxicity associated with the plasmid.

The toxicity mediated by IPTG induction of OGCP expression in *E. coli* BL21(DE3) in a liquid culture was investigated by plating the cells in the absence of selection pressure, in the presence of ampicillin, and in presence of both ampicillin and IPTG. After 30 minutes, the number of viable cells had decreased dramatically from 10⁸ to 10⁴ even in the absence of ampicillin (see Figure 1B). The absorbance of the culture was still increasing at this

juncture, and so the cells were dying but had not lysed. In the residual viable population, only 10% of the bacteria retained ampicillin resistance two hours after induction, and three hours later only 1% of the population was resistant to the antibiotic. Eight hours after induction, the number of viable cells equalled the number of cells calculated from the absorbance, showing that the culture now contained predominantly cells that had lost the plasmid.

The ampicillin-resistant cells were also resistant to IPTG (Figure 1B), and the colonies contained two sub-populations of larger and smaller sizes (Figure 1C). Neither of these phenomena has been described in previous investigations of this expression system (Studier *et al.*, 1990). In a separate control experiment, where no IPTG was added to the liquid culture, the plasmid was stable, and the number of viable cells was similar to the number of cells calculated from the absorbance. However, on plates, a small fraction of the population was again resistant to IPTG. In the uninduced liquid culture, the ratio of cells resistant to IPTG compared to the total number of cells was stable at around 3×10^{-3} , whereas induction of expression by addition of IPTG increased this ratio to 3×10^{-2} (Figure 1D). Therefore, the expression of the OGCP in *E. coli* BL21(DE3) grown in liquid cultures had apparently increased the number of colonies resistant to IPTG. This effect of IPTG is difficult to quantify as its addition to the culture leads to the death of wild-type cells, thereby selecting for mutants, but it remains possible that the induction of expression of the protein provokes an SOS response in the host (Studier *et al.*, 1990; Murli & Walker, 1993; Friedberg *et al.*, 1995).

This apparent increase in the frequency of mutants by over-expression of the OGCP on *E. coli* BL21(DE3) presented the opportunity to select mutant host strains that might be more tolerant to over-expression of the OGCP. The first round of selection was conducted with *E. coli* BL21(DE3) transformed with pMW7(OGCP). Four hours after induction, a 100-fold dilution of cells from the culture was plated on solid medium containing IPTG, giving sub-populations of large and small colonies as before (Figure 1C). Three large colonies and one small colony were examined for their ability to express the OGCP in liquid media. No OGCP was produced by cells grown from the large colonies, but a culture grown from the small colony was found to produce the OGCP and to continue growing in the presence of IPTG, eventually attaining a saturation absorbance similar to control cultures grown in the absence of inducer. The strain of cells from the small colony was named *E. coli* C41(DE3). Its phenotype was stable; it continued to give rise to small colonies in the presence of IPTG (Figure 2A), and to grow and to produce the OGCP in liquid cultures in the presence of inducer.

Plasmid pMW7(OGCP) was re-isolated from cells of *E. coli* C41(DE3), and was transformed back into *E. coli* BL21(DE3), restoring the toxic phenotype.

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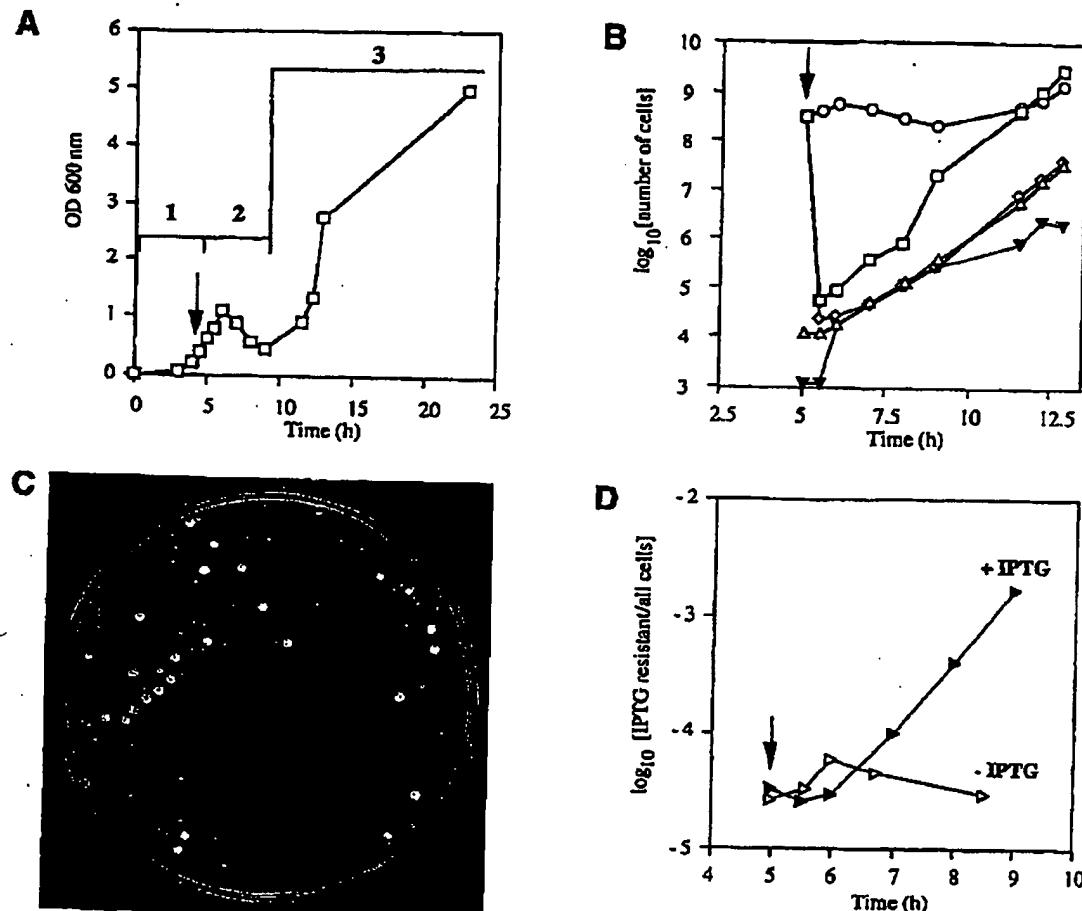
Protein Over-expression in *E. coli*

Figure 1. Effect of expression of bovine OGCP on the growth of *E. coli* BL21(DE3) host cells. In A, B and D, the vertical arrow indicates the addition of the inducer IPTG (final concentration 0.7 mM) to the liquid culture. A, A fresh colony of the host containing the plasmid pMW7(OGCP) was inoculated into 2 × TY medium (50 ml) supplemented with ampicillin (final concentration 50 µg/ml). Three phases of growth are marked: 1, pre-induction; 2, post-induction cell death; 3, overgrowth of the culture. B to D, Analysis of the bacterial population after induction of expression of the bovine OGCP. Portions (100 µl) of each dilution (1 in 10, 1 in 10², 1 in 10³, 1 in 10⁴ and 1 in 10⁵) were spread on three sets of agar plates, (0.7 mM, final concentration), IPTG and ampicillin (50 µg/ml), ampicillin alone, and no additives, respectively. The number of viable cells was determined by counting the colonies on the most suitable plate with 100 to 300 colonies per plate. In B, the analysis was performed on samples from A. The symbols used are as follows: (○) Number of cells calculated from the absorbance; (□) number of viable cells on 2 × TY plates; (◊) ampicillin-resistant colonies; (△) ampicillin and IPTG-resistant colonies; (▼) small colonies resistant to both ampicillin and IPTG ("small" "large" colonies). C, Large and small colony formation in the presence of ampicillin and IPTG of a sample of cells from a liquid culture taken 11.5 hours after induction by IPTG. D, The frequency of ampicillin and IPTG resistant colonies compared with the total population: (▷) non-induced culture; (▶) induced culture.

Strain *E. coli* C41(DE3) was cured of pMW7(OGCP) by growth in liquid medium in the absence of ampicillin. Each day, a portion of the culture was diluted 1000-fold, and plated out in the presence of IPTG and in the absence of ampicillin. After seven days, a large colony lacking the plasmid arose. Retransformation of cells from this colony with pMW7(OGCP) restored the ability to grow in the presence of IPTG in liquid culture and to over-express the OGCP. Therefore, the

mutation affecting over-expression of the OGCP is in strain C41(DE3), and not in the plasmid pMW7(OGCP).

Subsequently, it has proved to be possible to over-express many other proteins without toxic effects in *E. coli* C41(DE3) (see below). However, the toxicity of over-expression of other proteins, including the b subunit of *E. coli* F-ATPase, persisted in strain C41(DE3). Therefore, a second round of selection was conducted on *E. coli*

C41(DE3) transformed with pMW7(Ecb). From 15 small colonies arising by plating in the presence of IPTG, one was found that over-expressed subunit b and continued to grow after induction. This strain was named *E. coli* C43(DE3), and the additional mutation was again shown to be associated with the bacterial genome. Similar to strain C41(DE3), the phenotype of C43(DE3) is stable (see Figure 3A). In contrast to their toxic effects on *E. coli* BL21(DE3),

"empty" plasmids do not inhibit the growth of either the C41(DE3) or C43(DE3) strains.

It should be emphasized that the number of small colonies and the proportion of those small colonies that are competent for expression of a target protein, differ widely according to the toxicity of the expression plasmid. With relatively non-toxic plasmids such as pMW7(OGCP) and pMW7(GFP), encoding the green fluorescent protein, small

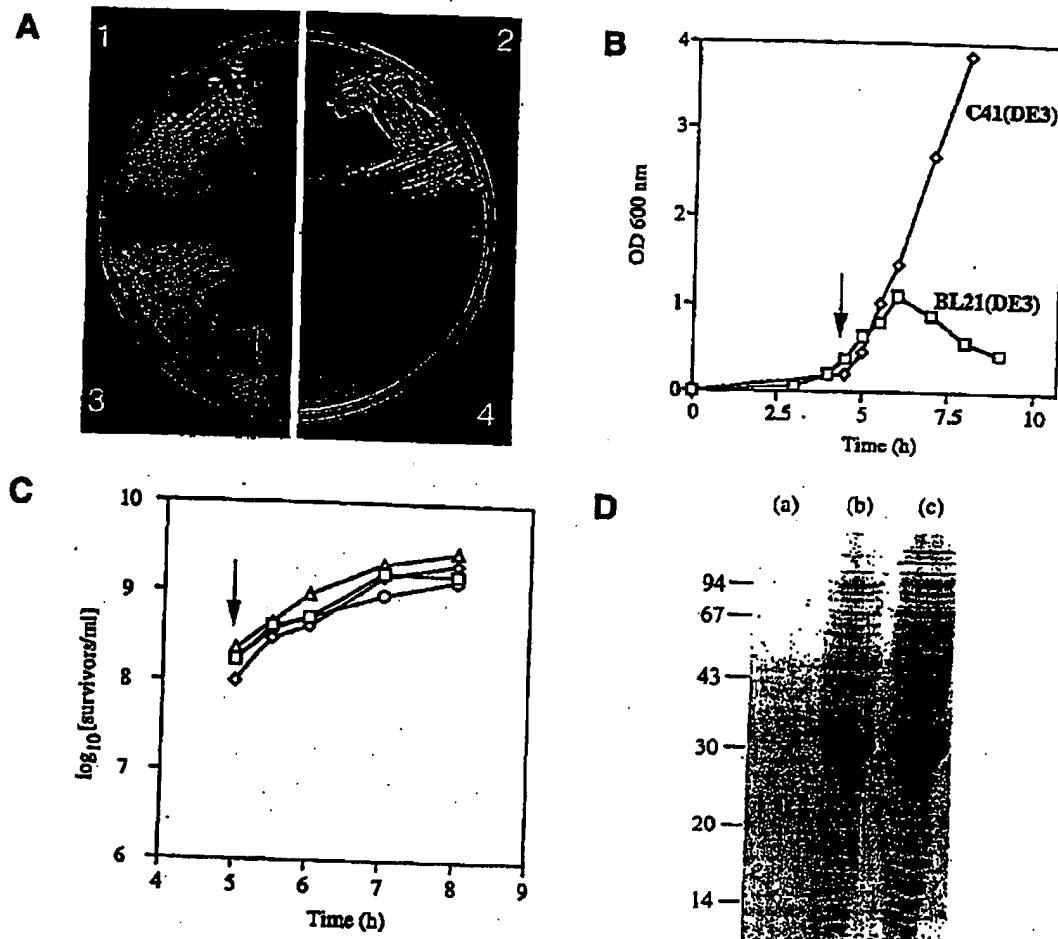


Figure 2. Comparison of the expression of the OGCP in *E. coli* BL21(DE3) and C41(DE3) hosts. A. Comparison of phenotypes of *E. coli* BL21(DE3) and mutant C41(DE3), both containing pMW7(OGCP). Quadrants 1 and 2, *E. coli* C41, of IPTG, respectively; quadrants 3 and 4, *E. coli* BL21(DE3) in the absence and presence of IPTG, respectively. B, Growth of the two strains containing pMW7(OGCP). C, Analysis of the bacterial population in the liquid culture (for details, see the legend to Figure 2). The arrows in B and C indicate the addition of inducer. (O) Number of cells calculated from the absorbance; (□) number of cells able to grow on 2 x TY plates; (○) ampicillin resistant colonies; (Δ) colonies resistant to both ampicillin and IPTG. D, Expression of the OGCP analysed by SDS-PAGE. The cultures were grown in 250 ml of broth. In both cases, the protein formed inclusion bodies in bacterial cytoplasm. They were each re-suspended in 4 ml of buffer and 1 μ l was analysed on the gel, which was stained with PAGE 83 dye. At the left-hand side, the positions of molecular mass markers are indicated (in kDa). Lane (a), OGCP expressed in *E. coli* BL21(DE3) three hours after induction; lane (b), OGCP expressed in C41(DE3) three hours after induction in medium lacking ampicillin; lane (c), OGCP expressed in C41(DE3) 18 hours after induction by IPTG added at the starting point of the culture.

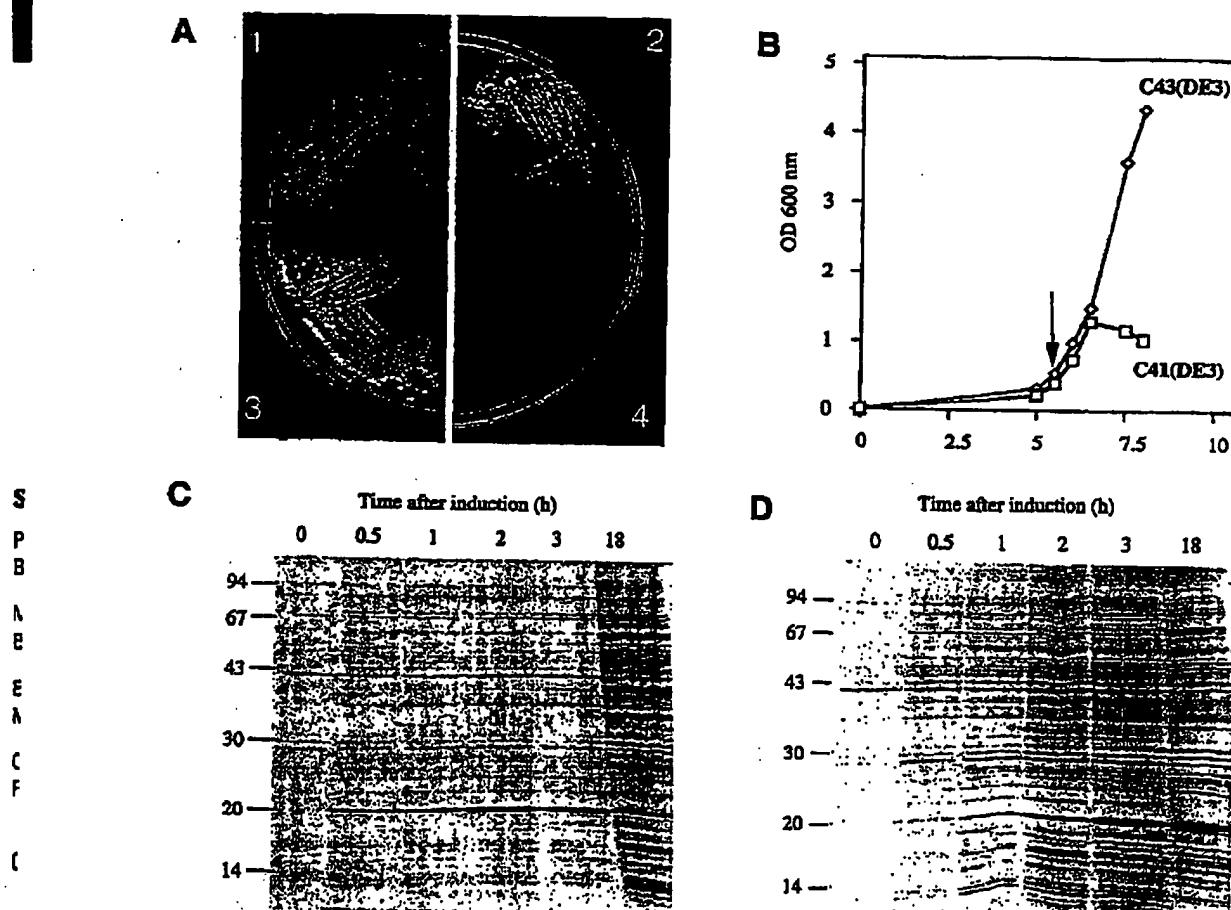


Figure 3. Comparison of the expression of subunit b of *E. coli* F-ATPase in *E. coli* C41(DE3) and C43(DE3) hosts. Freshly transformed colonies C41(DE3) and C43(DE3) each containing pMW7(Ecb) were inoculated into 2 x TY medium (50 ml) and grown at 37°C. A, Comparison of phenotypes of *E. coli* C41(DE3) and mutant C43(DE3), both containing pMW7(Ecb). Quadrants 1 and 2, *E. coli* C43(DE3), in the absence and presence of IPTG, respectively; quadrants 3 and 4, *E. coli* C41(DE3) in the absence and presence of IPTG, respectively. B, Growth curves of *E. coli* C41(DE3) and C43(DE3) expressing subunit b. The arrow indicates the induction of expression by IPTG. C and D, SDS-PAGE analysis of the expression of the b-subunit in *E. coli* C41(DE3) and C43(DE3), respectively. The equivalent of 5 µl of culture was analysed at the times indicated above each slot. On the left-hand side, the migration positions of standard proteins are indicated. The gel was stained with Coomassie 83 dye.

colonies competent for over-expression were common and easily identifiable, whereas with more toxic plasmids such as pMW7(Ecb), mutants of C41(DE3) expressing subunit b were rare.

The advantages of strains C41(DE3) and C43(DE3) as hosts for over-expression of the OGCP and subunit b of the F-ATPase, respectively, are illustrated in Figures 2 and 3. Both parental strains stopped growing after induction of expression, whereas the mutant hosts continued to grow to high cell densities (see Figures 2B and 3B). By analysis of the cell population in the culture after induction of over-expression (Figure 2C), it was apparent that pMW7(OGCP) remained stable in strain C41(DE3), and in addition, the number of

viable cells correlated with the number of cells calculated from the absorbance. In C41(DE3), at least ten times more OGCP was expressed than in BL21(DE3) (Figure 2D, lanes (a) and (b)). Moreover, strain C41(DE3) containing pMW7(OGCP) could be grown in 2 x TY broth, containing IPTG but lacking ampicillin, without overgrowth (Figure 2D, lane (c)). The final cell density in C41(DE3) was six times greater than in BL21(DE3), and therefore the amount of OGCP produced per cell is somewhat higher in C41(DE3) than in BL21(DE3).

The course of expression of the *E. coli* F-ATPase b subunit in C41(DE3) and C43(DE3) differed (see Figure 3C and D), the onset of protein production being delayed in C43(DE3) by about one hour

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Protein Over-expression in *E. coli*

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relative to C41(DE3). Three hours after induction, three times more protein had been produced in C43(DE3) than in C41(DE3); 15 hours later the amount of subunit b in C41(DE3) had decreased because the culture had become overgrown by cells that had lost expression capacity, as discussed above. Less subunit b was produced per cell in C43(DE3) than in C41(DE3), but the global amount of protein produced per litre of culture was higher in C43(DE3) because the cells continued to divide after induction of expression.

Transcription of the gene for the OGCP was compared in BL21(DE3) and C41(DE3) hosts (see Figure 4). The main RNA band had migrated further than 16 S ribosomal RNA to a position corresponding to an mRNA of about one kilobase, as expected for the OGCP. Three larger RNAs, also

detected with the OGCP probe, probably arise by the T7 RNA polymerase transcribing beyond the T7 transcriptional terminator, which is immediately after the OGCP gene in the plasmid. In longer exposures of the blot (not shown), similar bands could be seen in C41(DE3) also, but in relatively lower amounts compared with the main band in BL21(DE3). The basal level of OGCP mRNA synthesis in BL21(DE3) was five times higher than in C41(DE3) (see Figure 4B), and the maximal amount of OGCP mRNA synthesized after induction was about ten times greater in BL21(DE3) than in C41(DE3). Moreover, the maximum amount of OGCP mRNA appears to have been reached after 45 minutes in BL21(DE3) and at least 45 minutes later in C41(DE3).

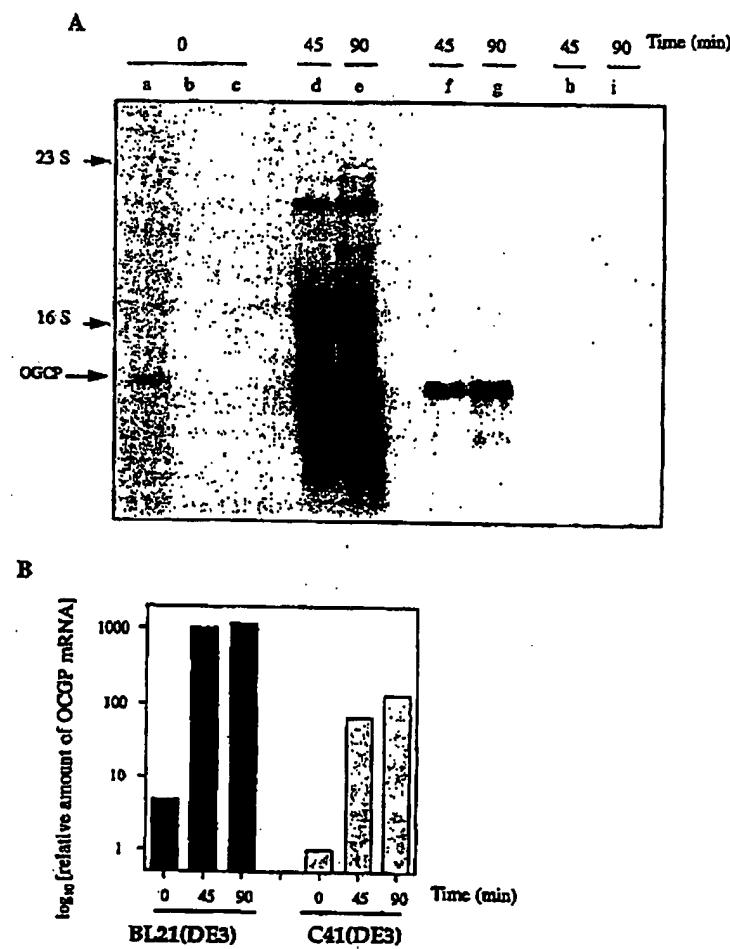


Figure 4. Analysis of transcripts of the OGCP in *E. coli* BL21(DE3) and C41(DE3). RNA samples from cells of *E. coli* BL21(DE3) and C41(DE3) (4 ml), both containing the expression plasmids for the OGCP and subunit b of *E. coli* F-ATPase, were prepared according to Ausubel *et al.* (1987) and Uzan *et al.* (1988), respectively. RNA (3 µg) was fractionated by electrophoresis under denaturing conditions in a 1% agarose gel and then transferred to a Hybond-N membrane. Pre-hybridization and hybridization of the membrane were carried out for 18 hours at 42°C. The DNA probe for the bovine OGCP, corresponding to its complete coding sequence, was amplified from a plasmid by PCR, and radio-labelled with [α -³²P]dCTP (50 µCi) by use of an oligonucleotide labelling kit (Pharmacia Biotech Ltd, St Albans, Herts, AL1 3AW, UK). The membrane was hybridized in the presence of the probe, washed twice at 42°C in 2 x SSC buffer containing 0.1% SDS, and twice at 65°C in 0.1 x SSC buffer containing 0.1% SDS (SSC buffer consists of 3 M sodium chloride and 0.3 M sodium citrate (pH 6.5)). The radioactivity on the membrane was measured by densitometry with a computing densitometer (Molecular Dynamics, model 300A with ImageQuant version 3.2 software) of a radioautograph exposed to Fuji RX film. A. Autoradiograph of the membrane after 15 minutes' exposure. Lanes a, d and e, RNA samples of BL21(DE3) expressing the OGCP; lanes b, f, and g, RNA samples of C41 expressing the OGCP; lanes c, h and i, control RNA samples from C41 expressing the *E. coli* F-ATPase b-subunit. Samples were taken at various times after induction, as shown on top. The migration positions of the OGCP mRNA, and of the 16 S and 23 S ribosomal RNAs are indicated on the left. B. Relative amounts of the OGCP mRNA, estimated by densitometry of the appropriate bands on two different exposures of the membrane. In C41(DE3), the signal at time zero was chosen as reference.

BL21(DE3) expressing the OGCP; lanes b, f, and g, RNA samples of C41 expressing the OGCP; lanes c, h and i, control RNA samples from C41 expressing the *E. coli* F-ATPase b-subunit. Samples were taken at various times after induction, as shown on top. The migration positions of the OGCP mRNA, and of the 16 S and 23 S ribosomal RNAs are indicated on the left. B. Relative amounts of the OGCP mRNA, estimated by densitometry of the appropriate bands on two different exposures of the membrane. In C41(DE3), the signal at time zero was chosen as reference.

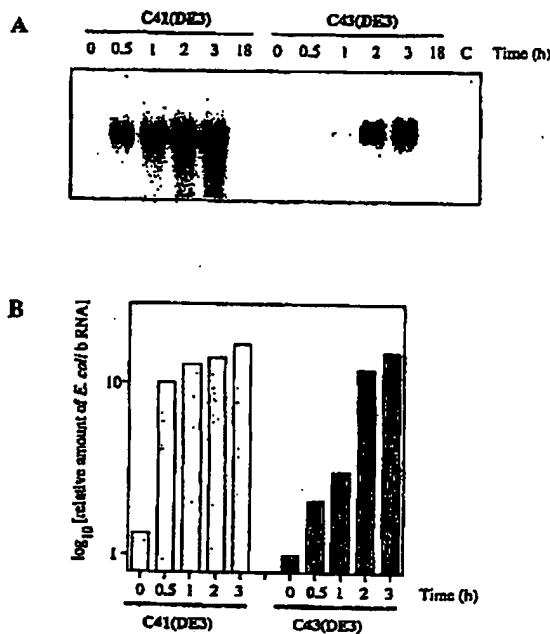


Figure 5. Analysis of transcripts of *E. coli* F-ATPase b subunit in C41(DE3) and C43(DE3). For experimental details, see legend to Figure 4. After hybridization of RNA samples with the probe consisting of the entire coding sequence of the b subunit, the membrane was exposed to an image plate for 18 hours, and the radioactivity was measured with a PhosphorImager (Molecular Dynamics, Chesham, Bucks, HP5 2PX, UK). A, Autoradiography of RNA samples from C41(DE3) and C43(DE3) at various times (shown on top) after induction of expression of F-ATPase subunit b. Lane C contains a control sample of RNA from C41(DE3) cells in which the OGCp had been over-expressed. B, Quantification of the mRNA samples in A. In C43(DE3), the signal at time zero was chosen as reference.

A similar comparison of the expression of the b subunit of the *E. coli* F-ATPase in C41(DE3) and C43(DE3) was also conducted (Figure 5). The final amount of mRNA for *E. coli* F-ATPase subunit b accumulated per cell after three hours was approximately the same in both strains, but the maximal level of this mRNA was attained in 30 minutes after induction in C41(DE3) and in two hours in C43(DE3). The basal level of expression was slightly lower in C43(DE3) than in C41(DE3).

These studies indicate strongly that a major component of toxicity of protein over-expression in *E. coli*-T7 RNA polymerase systems is linked to transcription of the target gene and, together with published work discussed below, they suggest that the toxicity probably arises from the uncoupling of transcription from translation. The T7 RNA polymerase transcribes DNA about seven times faster than *E. coli* RNA polymerase, and therefore

transcription outstrips translation, permitting unstable naked RNA stretches to form (Iost & Dreyfus, 1995; Makarova *et al.*, 1995). By an unknown mechanism, over-expression of either β -galactosidase or an inactive form of elongation factor Tu have been shown to lead to destruction of ribosomal RNAs, and the ensuing lethal effects of over-expression (Dong *et al.*, 1995). Experiments described above are consistent with this mechanism of lethality of protein over-expression. Strain BL21(DE3) containing the expression plasmid pMW7(OGCP) produced a large amount of the cognate mRNA from the plasmid, whilst at the same time the target protein was present in the cells at rather low levels. In contrast, in strain C41(DE3) the same transcript was made more slowly, and despite the maximal level being ten times lower than in BL21(DE3), more of the target protein was synthesized in C41(DE3). Similar effects were noted by comparison of the expression of subunit b of *E. coli* F-ATPase in C41(DE3) and C43(DE3).

At present, the locations of the mutations in C41(DE3) and C43(DE3) are not known, but plausible hypotheses concerning the C41(DE3) mutation are that either it affects the activity of the T7 RNA polymerase or that it reduces the amount of polymerase produced. Both effects would probably help to prevent uncoupling of transcription and translation. It is noteworthy that a mutant of T7 RNA polymerase able to transcribe three times more slowly than the wild-type enzyme has been shown to yield about four times more β -galactosidase from an appropriate expression vector (Makarova *et al.*, 1995). The C43(DE3) mutation may also be helping to avoid uncoupling of transcription and translation, but, in addition to delaying the onset of transcription, it also appears to affect the folding and insertion of subunit b into the bacterial membrane. In C41(DE3) the *E. coli* F-ATPase b subunit accumulates in a form that is difficult to solubilize in the detergent lauryldimethylamine oxide, and it may be misfolded, whereas in C43(DE3) it is inserted into the membrane and can be readily extracted with the detergent (I. Arechaga, B.M. & J.E.W., unpublished results).

The expression levels of a variety of proteins (seven membrane proteins, ten globular proteins; see Table 1) in BL21(DE3) were compared with the levels achieved in either C41(DE3) or C43(DE3) hosts. For all seven of the membrane proteins, and particularly for the alanine-H⁺ transporter and the *E. coli* F-ATPase subunits b and c, expression in the mutant hosts was improved over BL21(DE3). In all three of these latter examples, the induction of the expression both on plates and in liquid media was toxic to C41(DE3) but not to C43(DE3). The gene 10a-alanine-H⁺ carrier fusion was very well expressed in C43(DE3), and 79 mg of protein were obtained per litre of culture (Table 1). Significant improvements in expression level were also obtained with the ADP/ATP and phosphate carriers in C41(DE3).

A general improvement in the expression of the globular proteins (see Table 1) was also found in mutant host C41(DE3), including proteins that were well expressed as well as others that were poorly expressed in BL21(DE3). The GFP provides a typical example of the former category. Although it was expressed at 37 mg per litre of culture in BL21(DE3), a four times higher level of expression was obtained in C41(DE3) (see Table 1). The γ -subunit of bovine ATPase provides an example of the second category. Cells of BL21(DE3) containing pMW7(γ) stopped growing at low density, and the γ -subunit was undetectable by SDS-PAGE analysis of the cells, whereas in C41(DE3) the cells continued to divide, grew to high density and produced a large amount of the γ -subunit (see Table 1).

The RNA polymerase of phage T7 is inhibited by the phage's lysozyme (Moffatt & Studier, 1987), and therefore, co-transformation of a plasmid encoding the lysozyme (pLysS and pLysE) with the plasmid containing the target protein has been advocated as a means of suppressing toxic effects brought about by basal level expression of proteins (Studier *et al.*, 1990). This stratagem has been found to be helpful in some cases of relatively mild toxicity. However, co-transformation of pLysS with pMW7(γ) or with pMW7(GFP) did not suppress their toxicities on agar plates in the presence of IPTG. In liquid media, the level of expression of both proteins was somewhat higher in BL21(DE3) in the presence of pLysS than in its absence, but the level of expression of both proteins in C41(DE3) was at least twice the level obtained in co-transformed BL21(DE3) cells. Therefore, at least in these two examples, C41(DE3) is superior to BL21(DE3)-pLysS as a host for over-expression of proteins. The combination of C41(DE3) with pLysS may be advantageous, but this possibility has not been investigated systematically so far.

To date, the usage of strain C43(DE3) for expression of other toxic proteins has been explored in only a small number of examples (the F-ATPase β and γ subunits, the alanine-H⁺ carrier). One example (an RNase) has been encountered where an expression vector that was toxic in C41(DE3) remained toxic in C43(DE3) also (unpublished results). A number of other examples show that, if the protein is already expressed without toxic effect in C41(DE3), then no additional benefit derives from over-expression of the same protein in C43(DE3). Therefore, it appears that C43(DE3) has a more restricted utility than C41(DE3).

Although the selection procedure presented here is empirical, it has the advantage that it encompasses the entire complexity of the biology of the expression system, and it has provided an efficient means of modifying it. The method takes advantage of a population of bacteria selected in the presence of both IPTG and ampicillin, which had been described incorrectly as only containing cells that have lost the ability to express the target DNA (Studier *et al.*, 1990). As we have shown, two

sub-populations giving rise to large and small colonies are present, and the latter contains cells that over-express the target protein better than the original host. Therefore, the procedure allows the expression system to be adapted and optimized for the expression of a particular protein, and it may be beneficial in other instances (including both globular and membrane proteins) to use the selection protocol to select a wider range of host strains derived, for example, from BL21(DE3), C41(DE3) and C43(DE3). In this way it may be possible to tailor the expression system by selection and thereby, for example, to prevent the formation of inclusion bodies, and to overcome toxic effects of various severities and origins.

Finally, it should be noted that removal of the toxic effects of an expression plasmid will not automatically guarantee that the protein is produced in large amounts, and to achieve this objective it may be necessary, for example, additionally to prevent mRNA degradation, to remove undesirable features in the coding sequence that impede translation (Kane, 1995), or to prevent proteolytic degradation.

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References

- Ausubel, F. M., Brent, R., Kingston, R. E., Seidman, D. D., Smith, J. G., Struhl, J. A. & Struhl, K. (1987). In *Current Protocols in Molecular Biology*, John Wiley & Sons Inc, New York.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, 263, 802-805.
- Collinson, I. R., van Raaij, M. J., Runswick, M. J., Fearnley, I. M., Skehel, J. M., Orriss, G., Miroux, B. & Walker, J. E. (1994). ATP synthase from bovine heart mitochondria: *in vitro* assembly of a stalk complex in the presence of F₁-ATPase and in its absence. *J. Mol. Biol.* 242, 408-421.
- de Boer, P. A. J., Crossley, R. E. & Rothfield, L. I. (1988). Isolation and properties of *min B*, a complex genetic locus involved in correct placement of the division site in *Escherichia coli*. *J. Bacteriol.* 170, 2106-2112.
- Doherty, A. J., Connolly, B. A. & Worrall, A. F. (1993). Overproduction of the toxic protein bovine pancreatic DNase I in *Escherichia coli* using a tightly controlled T7 promoter based vector. *Genic*, 136, 337-340.
- Dong, H., Nilsson, L. & Kurland, C. G. (1995). Gratuious overexpression of genes in *Escherichia coli* leads to growth inhibition and ribosome destruction. *J. Bacteriol.* 177, 1497-1504.
- Fiermonte, G., Walker, J. E. & Palmieri, F. (1993). Abundant bacterial expression and reconstitution of

FROM BIOMEDICAL INFORMATION SERVICE

(FRI) 5. 11' 01 15:11/ST. 15:02/NO. 4862641830 P 14

an intrinsic membrane transport protein from bovine mitochondria. *Biochem. J.* 294, 293-299.

Fillingame, R. H. (1990). Molecular mechanics of ATP synthesis by F_1F_0 -type H^+ -transporting ATPases. *The Bacteria*, 12, 345-391.

Friedberg, E. C., Walker, G. C. & Siede, W. (1995). In *DNA Repair and Mutagenesis*, ASM Press, Washington, DC.

George, J. W., Brosh, R. M., Jr & Matson, S. W. (1994). A dominant negative allele of the *Escherichia coli* *uvrD* gene encoding DNA helicase II. *J. Mol. Biol.* 235, 424-435.

Grisshammer, R. & Tate, C. G. (1995). Overexpression of integral membrane proteins for structural studies. *Quart. Rev. Biophys.* 28, 315-422.

Gutzman, L. M., Barondess, J. J. & Beckwith, J. (1992). Fts L, an essential cytoplasmic membrane protein involved in cell division in *Escherichia coli*. *J. Bacteriol.* 174, 7716-7728.

Hockney, R. C. (1994). Recent developments in heterologous protein production in *Escherichia coli*. *Trends Biotechnol.* 12, 456-463.

Ilost, I. & Dreyfus, M. (1995). The stability of *Escherichia coli* lacZ mRNA depends upon the simultaneity of its synthesis and translation. *EMBO J.* 14, 3252-3261.

Kamata, H., Akiyama, S., Morosawa, H., Ohta, T., Hamamoto, T., Kambe, T., Kagawa, Y. & Hirata, H. (1992). Primary structure of the alanine carrier protein of thermophilic bacterium PS3. *J. Biol. Chem.* 267, 21650-21655.

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6, 494-500.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680-685.

Makarova, O. V., Makarov, E. M., Sousa, R. & Dreyfus, M. (1995). Transcribing of *Escherichia coli* genes with mutant T7 RNA polymerases: stability of lacZ mRNA inversely correlates with polymerase speed. *Proc. Natl. Acad. Sci. USA*, 92, 12250-12254.

Moffatt, B. A. & Studier, F. W. (1987). T7 lysozyme inhibits transcription by T7 RNA polymerase. *Cell*, 49, 221-227.

Murli, S. & Walker, G. C. (1993). SOS mutagenesis. *Curr. Opin. Genet. Dev.* 3, 719-725.

Orriss, G. L., Runswick, M. J., Collinson, I. R., Miroux, B., Fearnley, I. M., Skehel, J. M. & Walker, J. E. (1996). The δ - and ϵ -subunits of bovine F_1 -ATPase interact to form a heterodimeric subcomplex. *Biochem. J.* 314, 695-700.

St Johnston, D., Beuchle, D. & Nüsslein-Volhard, C. (1991). *Staußen*, a gene required to localize maternal RNAs in the *Drosophila* egg. *Cell*, 66, 51-63.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185, 60-89.

Uzan, M., Favre, R. & Brody, E. (1988). A nuclease that cuts specifically in the ribosome binding site some T4 mRNAs. *Proc. Natl. Acad. Sci. USA*, 85, 8895-8899.

Walker, J. E. & Runswick, M. J. (1993). The mitochondrial transport protein super-family. *J. Bioenerget. Biomembr.* 25, 435-467.

Walker, J. E., Runswick, M. J. & Poulter, L. (1987). ATP synthase from bovine mitochondria: characterization and sequence analysis of two membrane associated subunits and of their corresponding cDNAs. *J. Mol. Biol.* 197, 89-100.

Way, M., Pope, B., Hawkins, M. & Weeds, A. G. (1990). Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J.* 9, 4103-4109.

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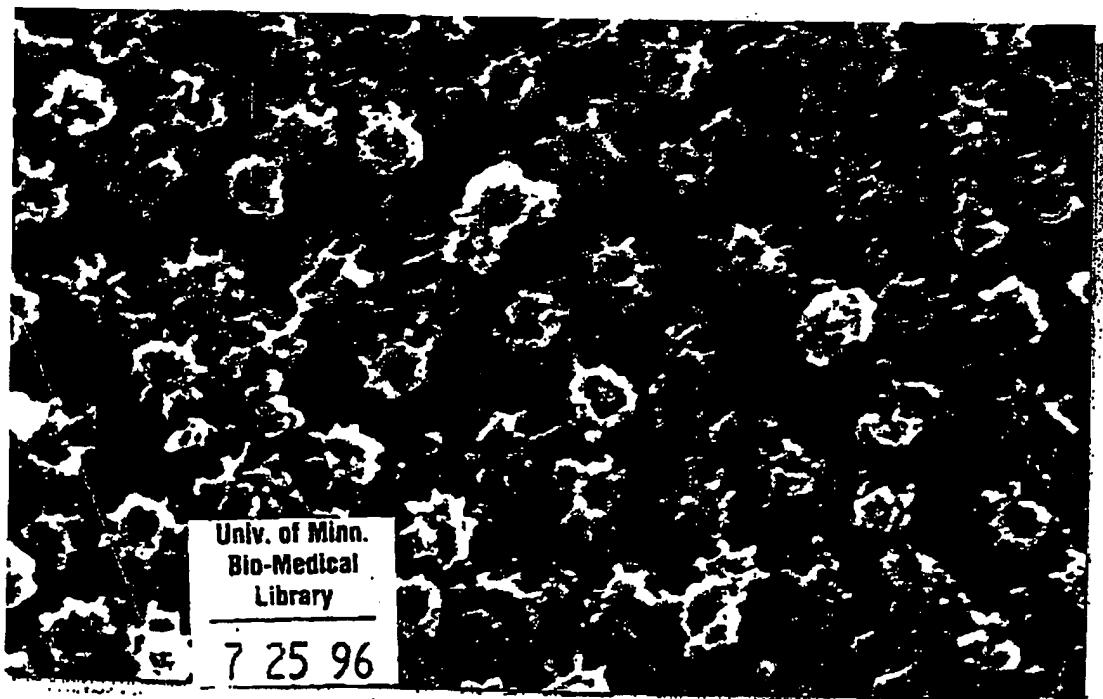
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Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

ANT1m ATGGGTGATCACCGCTTGGACCTTCCTAAGGACTTCCTGGCCGGGGGCTCGCCGCTCCAAGACGGCGGTGGC 80
ANT2m ATGACAGATGGCGCTGTGCTCTCGCAAGGACTTCCTGGCAGGTGGAGTGCGCGCAGCCATCTCCAAGACGGCGGTAGC 80
ANT3m ATGACGGAAACAGGOCATCTCTCGCCAAGACTTCCTGGCCGGAGGCATCGCCGCCATCTCCAAGACGGCGGTGGC 80

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ANT3m TCGATCGAGCGGGTCAAGCTGCTGCTGCAGGTCCAGCAAGCCAGCAAGCAGATCGCGCGACAAGCAGTACAAAGGCA 160

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ANT3m GCAGTTCTGGAGGTACTTTGGGGGAAACCTGGGTCCGGGGTGGCGCGCGACCTCCCTTGCTTGTGTACCCGC 400

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ANT3m TGGATTTGCCAGAACCGCCTGGCAGCGAGGTGGGAAAGTGGAGGACAGAGCGGGAGTTCCGAGGCCTGGAGACTGC 480

Fig. 1A

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

ANT1m	ATCA <u>AA</u> CAAGATCT <u>CA</u> AGTCTGATGGC <u>GG</u> AGGGGG <u>CT</u> T <u>AC</u> CCAGGG <u>TT</u> TCACGTC <u>CT</u> GT <u>CC</u> AGGCAT <u>CT</u> AT <u>AT</u> CTA	557
ANT2m	CTGG <u>TT</u> AA <u>AG</u> ATCT <u>AC</u> AA <u>AT</u> CTGATGG <u>GA</u> TT <u>AA</u> GGGC <u>CT</u> GT <u>AC</u> CA <u>AG</u> CT <u>TT</u> AA <u>AC</u> GT <u>GT</u> CT <u>GT</u> GC <u>AG</u> GG <u>TT</u> AT <u>AT</u> CT <u>AT</u> CTA	560
ANT3m	CTGG <u>TT</u> AA <u>AG</u> AT <u>AC</u> CA <u>AG</u> TO <u>GA</u> GG <u>GC</u> AT <u>CG</u> GGGG <u>CT</u> GT <u>AC</u> CA <u>GG</u> GG <u>CT</u> CA <u>GT</u> <u>GT</u> CT <u>CG</u> GT <u>CA</u> GG <u>GC</u> AT <u>CA</u> CT <u>CT</u> TA	560
ANT1m	<u>TA</u> AG <u>AG</u> GT <u>GC</u> CT <u>AC</u> TT <u>CG</u> G <u>AG</u> T <u>CT</u> AT <u>GA</u> TA <u>CT</u> G <u>CC</u> A <u>AG</u> GG <u>GA</u> T <u>G</u> C <u>G</u> CT <u>GC</u> CT <u>GA</u> CCCC <u>CA</u> AG <u>AA</u> AC <u>GT</u> <u>GC</u> AC <u>AT</u> <u>TT</u> <u>GT</u> G <u>AG</u> GT	637
ANT2m	CC <u>GA</u> GG <u>GG</u> C <u>CT</u> ACT <u>TC</u> GG <u>TA</u> CT <u>AT</u> GA <u>AC</u> CT <u>GC</u> <u>AA</u> AG <u>GG</u> AA <u>AT</u> GC <u>TT</u> CC <u>GG</u> <u>AT</u> CC <u>CA</u> AG <u>AA</u> AC <u>AC</u> <u>GT</u> <u>AC</u> AT <u>CG</u> TC <u>AT</u> <u>CA</u> <u>AG</u> GT	640
ANT3m	CC <u>GG</u> GG <u>GG</u> C <u>CT</u> ACT <u>TC</u> GG <u>CG</u> <u>GT</u> <u>GT</u> <u>AC</u> GA <u>TA</u> CG <u>CC</u> A <u>AG</u> GG <u>GA</u> T <u>G</u> C <u>TC</u> CG <u>GG</u> AC <u>CC</u> CA <u>AG</u> AA <u>AC</u> AC <u>CG</u> AC <u>AT</u> CG <u>TC</u> <u>GT</u> <u>GT</u> G <u>AG</u> GT	640
ANT1m	GG <u>AT</u> GT <u>GA</u> <u>TT</u> GC <u>CC</u> A <u>GA</u> <u>AC</u> <u>GT</u> <u>GT</u> G <u>AC</u> GG <u>GC</u> <u>AG</u> <u>GT</u> <u>CG</u> <u>GT</u> <u>GT</u> CC <u>CT</u> AC <u>CC</u> CT <u>TT</u> <u>GA</u> AC <u>AT</u> GT <u>TT</u> CG <u>TC</u> <u>GT</u> <u>GT</u> <u>AG</u> AT <u>GT</u> G <u>AT</u> GT	717
ANT2m	GG <u>AT</u> GT <u>GA</u> <u>TC</u> GC <u>CA</u> <u>GA</u> <u>AC</u> <u>GT</u> <u>GT</u> <u>AC</u> <u>GT</u> <u>GT</u> <u>GC</u> <u>CC</u> GG <u>GT</u> <u>TC</u> AC <u>CT</u> <u>TC</u> TA <u>CC</u> <u>AT</u> <u>TT</u> <u>GA</u> AC <u>AT</u> GT <u>TT</u> CG <u>CC</u> GG <u>GC</u> <u>AT</u> GT <u>GA</u> <u>AT</u> GT	720
ANT3m	GG <u>AT</u> GT <u>GA</u> <u>TC</u> GC <u>CA</u> <u>GA</u> <u>AC</u> <u>GT</u> <u>GT</u> <u>GA</u> <u>CG</u> <u>GG</u> <u>CG</u> <u>GT</u> <u>GG</u> <u>CC</u> GG <u>CG</u> <u>GT</u> <u>GT</u> <u>GT</u> <u>CC</u> CT <u>AC</u> <u>CC</u> <u>TT</u> <u>GA</u> AC <u>AC</u> <u>GT</u> <u>GC</u> <u>CC</u> GG <u>GC</u> <u>AT</u> GT <u>GA</u> <u>AT</u> GT	720
ANT1m	CA <u>GT</u> CC <u>GG</u> <u>GC</u> <u>GG</u> <u>AA</u> AG <u>GG</u> <u>GG</u> <u>GC</u> <u>GA</u> <u>AT</u> <u>AT</u> <u>GT</u> <u>AC</u> AC <u>GG</u> <u>GC</u> <u>AC</u> <u>GT</u> <u>GT</u> <u>AC</u> GT <u>CG</u> <u>TC</u> GG <u>AG</u> GA <u>AG</u> AT <u>GT</u> <u>GA</u> <u>AA</u> AG <u>AC</u> <u>GA</u> AG <u>GG</u> <u>AC</u>	797
ANT2m	CA <u>GT</u> <u>CA</u> <u>GG</u> <u>GC</u> <u>CA</u> <u>AA</u> AG <u>GG</u> <u>AA</u> <u>AC</u> <u>GT</u> <u>GA</u> <u>AT</u> <u>CT</u> <u>AT</u> <u>GT</u> <u>AC</u> AT <u>CA</u> <u>GT</u> <u>AC</u> <u>AC</u> <u>GG</u> <u>CA</u> <u>CG</u> <u>GT</u> <u>GT</u> <u>AC</u> GT <u>CG</u> <u>TC</u> <u>GG</u> <u>CG</u> <u>GA</u> <u>AG</u> AT <u>GT</u> <u>GT</u> <u>GA</u> AG <u>GG</u> <u>AG</u>	800
ANT3m	CA <u>GT</u> CC <u>GG</u> <u>GC</u> <u>CA</u> <u>AA</u> AG <u>GG</u> <u>AG</u> <u>GT</u> <u>GA</u> <u>AT</u> <u>CT</u> <u>AT</u> <u>GT</u> <u>AC</u> AC <u>GG</u> <u>GC</u> <u>AC</u> <u>GT</u> <u>GT</u> <u>AC</u> GT <u>GT</u> <u>GG</u> <u>AG</u> GA <u>AG</u> AT <u>CT</u> <u>CA</u> <u>AG</u> AG <u>AT</u> <u>GT</u> <u>AG</u> <u>GG</u> <u>GG</u>	800
ANT1m	CA <u>AG</u> GC <u>CT</u> <u>CT</u> CA <u>AA</u> <u>GG</u> <u>GT</u> <u>GC</u> <u>GT</u> <u>GG</u> <u>TC</u> CA <u>AT</u> <u>GT</u> <u>GT</u> <u>GT</u> <u>AG</u> AG <u>GG</u> <u>CA</u> <u>GG</u> <u>GC</u> <u>GG</u> <u>GT</u> <u>GT</u> <u>CT</u> <u>TT</u> <u>GT</u> <u>AT</u> <u>GT</u> <u>GT</u> <u>GT</u> <u>AT</u> GT <u>G</u> AG <u>AG</u>	877
ANT2m	CA <u>AA</u> <u>GG</u> <u>GT</u> <u>TT</u> <u>GT</u> <u>TC</u> AA <u>GG</u> <u>GT</u> <u>GC</u> <u>AT</u> <u>GG</u> <u>TC</u> CA <u>AT</u> <u>GT</u> <u>GT</u> <u>GT</u> <u>AG</u> AG <u>GG</u> <u>CA</u> <u>GG</u> <u>GC</u> <u>GG</u> <u>GT</u> <u>GT</u> <u>CT</u> <u>TT</u> <u>GT</u> <u>AT</u> <u>GT</u> <u>GA</u> <u>AA</u>	880
ANT3m	CA <u>AG</u> GC <u>CT</u> <u>CT</u> CA <u>AA</u> <u>GG</u> <u>GT</u> <u>GC</u> <u>GT</u> <u>GG</u> <u>TC</u> CA <u>AA</u> <u>GT</u> <u>GT</u> <u>GT</u> <u>GG</u> <u>GC</u> <u>GG</u> <u>GC</u> <u>GG</u> <u>GT</u> <u>GT</u> <u>CT</u> <u>GT</u> <u>GG</u> <u>GT</u> <u>GT</u> <u>GA</u> <u>AG</u> <u>AG</u>	880
ANT1m	T <u>CA</u> <u>AA</u> <u>AA</u> <u>AA</u> <u>AT</u> <u>GT</u> <u>CT</u> <u>AA</u> 894	
ANT2m	T <u>CA</u> <u>AG</u> <u>AA</u> <u>GT</u> <u>AC</u> <u>AC</u> <u>AT</u> <u>AA</u> 897	
ANT3m	T <u>CA</u> <u>AG</u> <u>AA</u> <u>AG</u> <u>GT</u> <u>GT</u> <u>AT</u> <u>CT</u> <u>AA</u> 897	

Fig. 1B

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

HANT1p	M QDIAV ISFL KDFLAG AVAAAV SKTAVAPIERVKLLLQVQHASKQ I A EKQ	50
HANT2p	MTDAAV ISFAKDFLAGG VAA ISK TAVAPIERVKLLLQVQHASKQ I T ADKQ	50
HANT3p	M TEQAV ISFAKDFLAGG I VAA ISK TAVAPIERVKLLLQVQHASKQ I A ADKQ	50
HANT1p	YKG I DCVVRIPKE QG I LSFWRGNLANVIRYFPTQALNFAFKDKY KQ I FL	100
HANT2p	YKG I DCVVRIPKE QE VLSFWRGNLANVIRYFPTQALNFAFKDKY KQ I FL	100
HANT3p	YKG I DQ I VRIPKE QG VLSFWRGNLANVIRYFPTQALNFAFKDKY KQ I FL	100
HANT1p	GGV D R I QFWRYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVG R R -A	149
HANT2p	GGV D K R I QFW I YFAGNLASGGAAGATSLCFVYPLDFARTRLAADVG K A G A	150
HANT3p	GGV D K H T Q F WRYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVG K S G I	150
HANT1p	D E F E GLG D G I I K I F K S D G I R G L YQGFNVSVQ III YRAAYFGVYDTAKG	199
HANT2p	EREF R G L G D C L M K I M K S D G I K G L YQGFNVSVQ III YRAAYFG V D T A K G	200
HANT3p	EREF R G L G D C L M K I T K S D G I R G L YQGFNVSVQ III YRAAYFGVYDTAKG	200
HANT1p	MLPDPK N V H I E V S W M I A S V TAVAGL I SYPFD T V R R M M Q S G R K G A I M	249
HANT2p	MLPDPK N T H I V I S W M I A Q V TAVAGL I SYPFD T V R R M M Q S G R K G I D I M	250
HANT3p	MLPDPK N T H I V V S W M I A Q V TAVAG V SYPFD T V R R M M Q S G R K G A I M	250
HANT1p	YTGT V D C W R K I A D E Q A K A F K G A W N V L R G M G A F V L V L Y D E I K K V	298
HANT2p	YTGT D C W R K I A R E D E G G K A F K G A W N V L R G M G A F V L V L Y D E I K K Y T	299
HANT3p	YTGT V D C W R K I F R D E G G K A F K G A W N V L R G M G A F V L V L Y D E I K K V I	299

Fig. 2

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

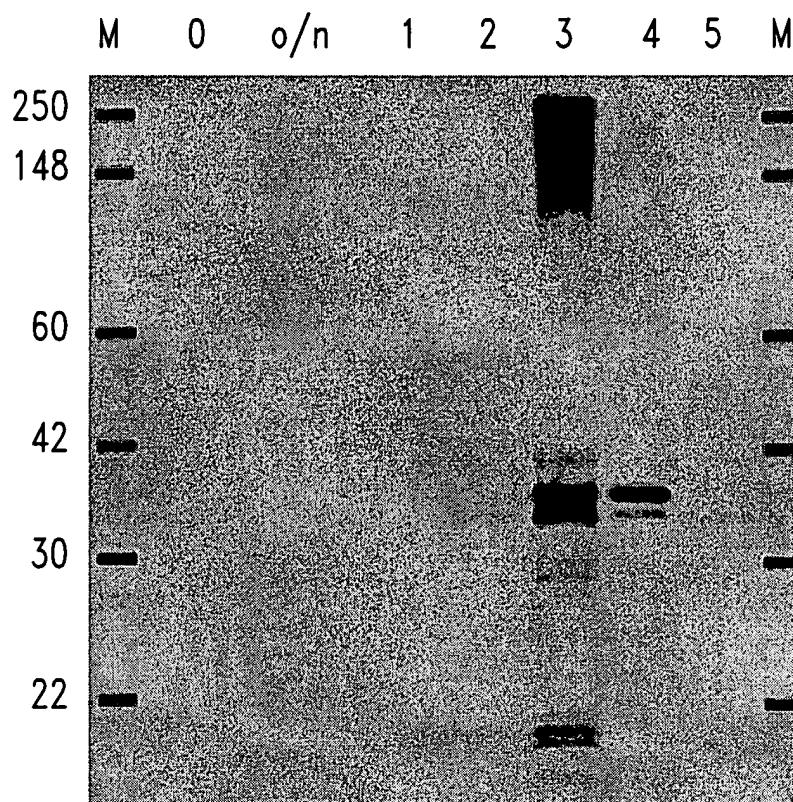


Fig. 3

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"
Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

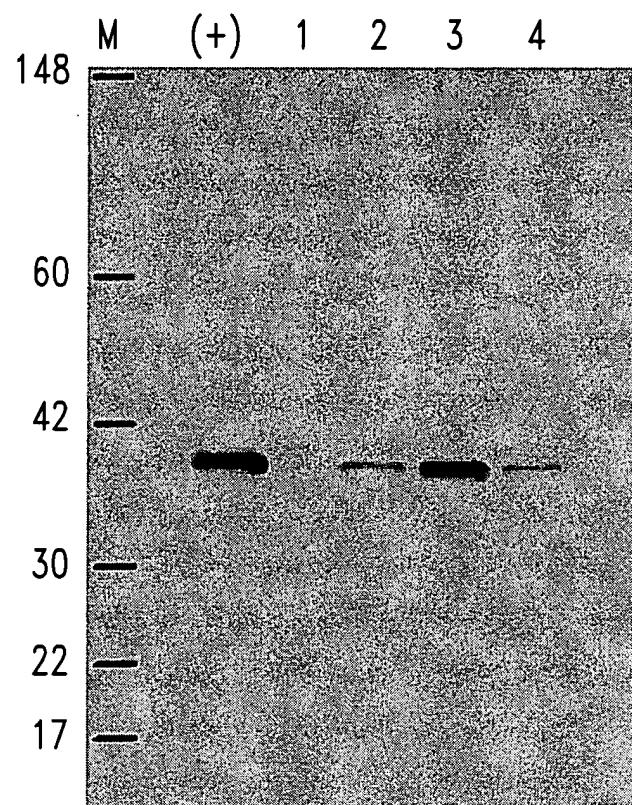


Fig. 4

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

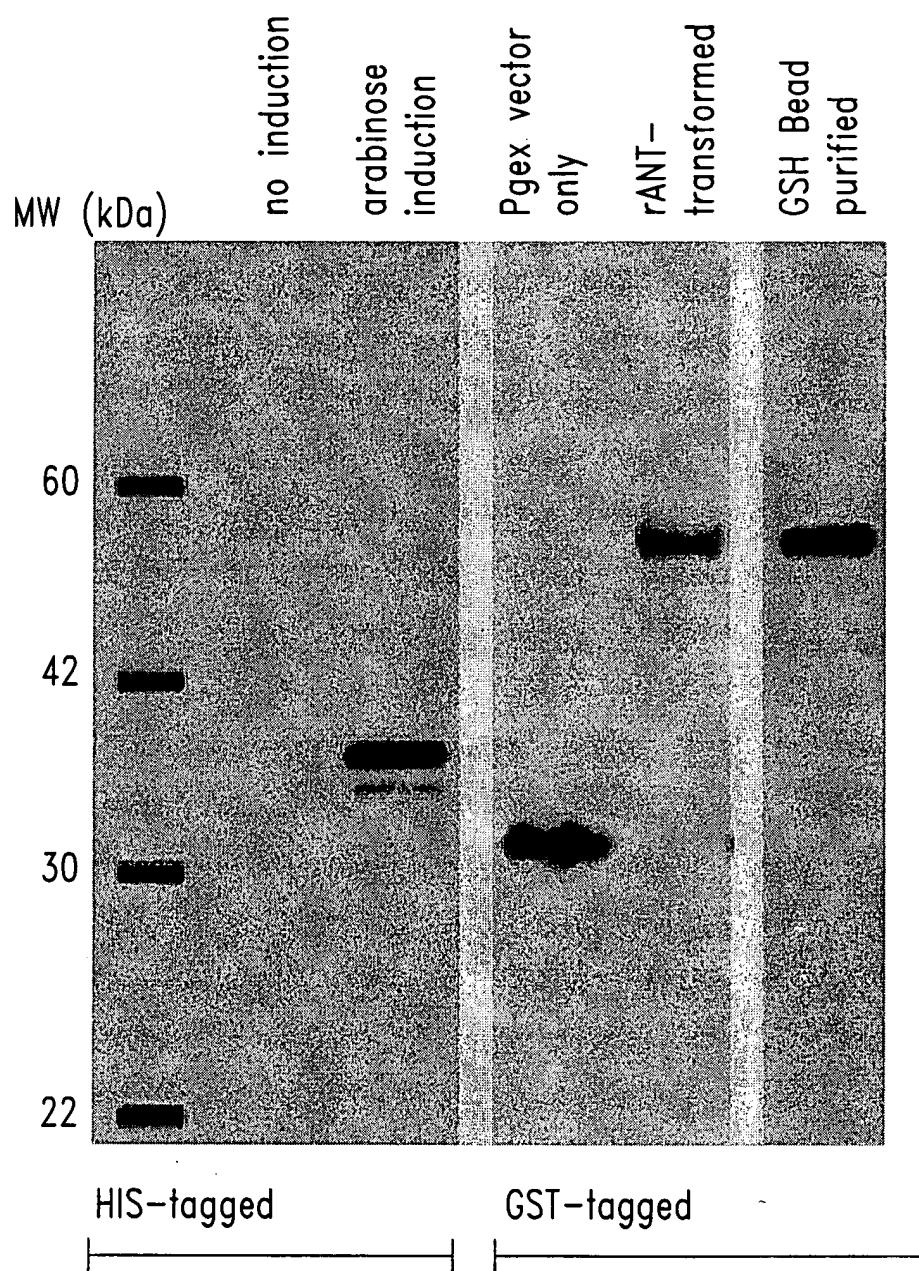


Fig. 5

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

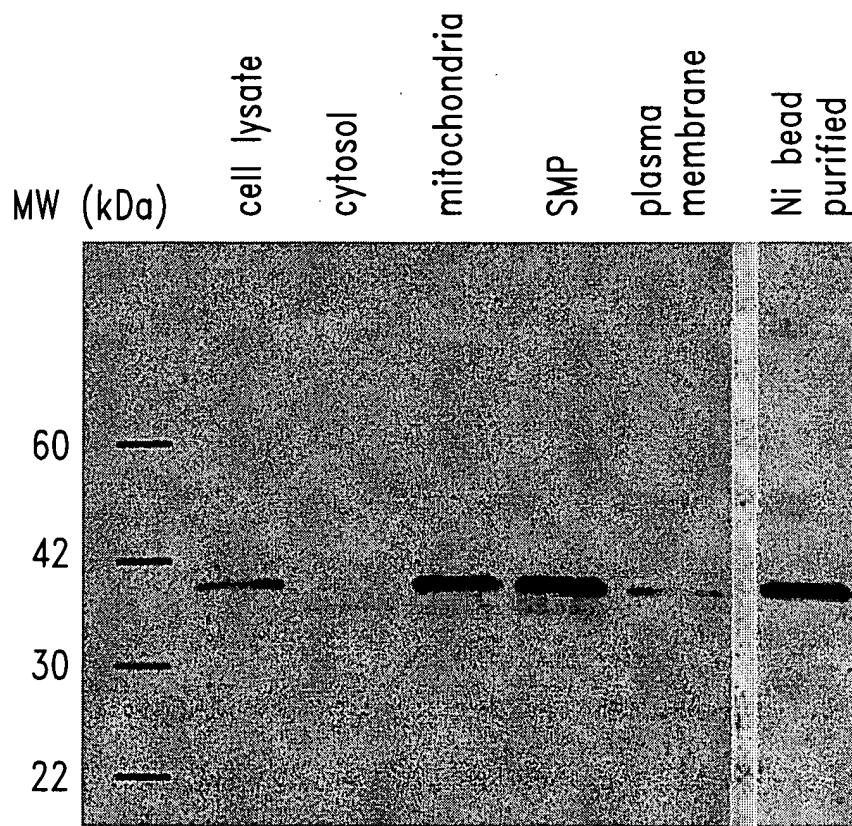


Fig. 6

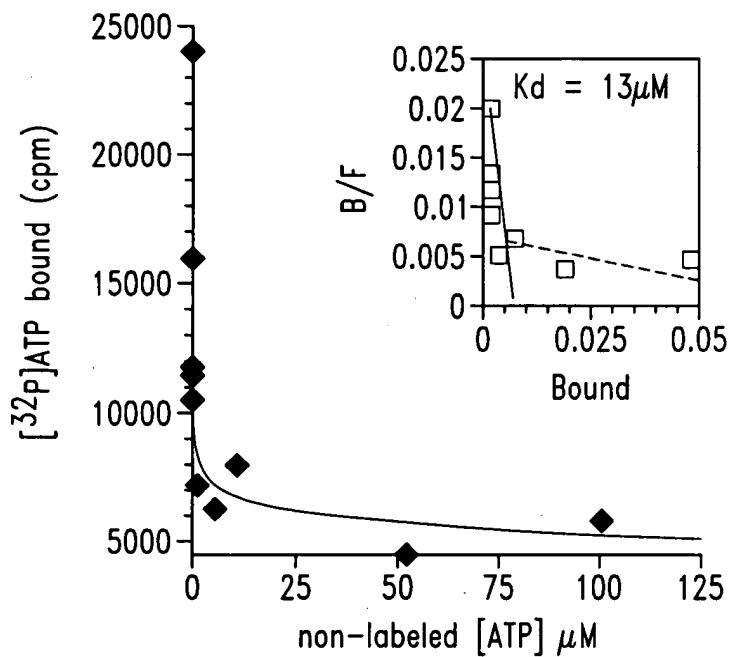


Fig. 7

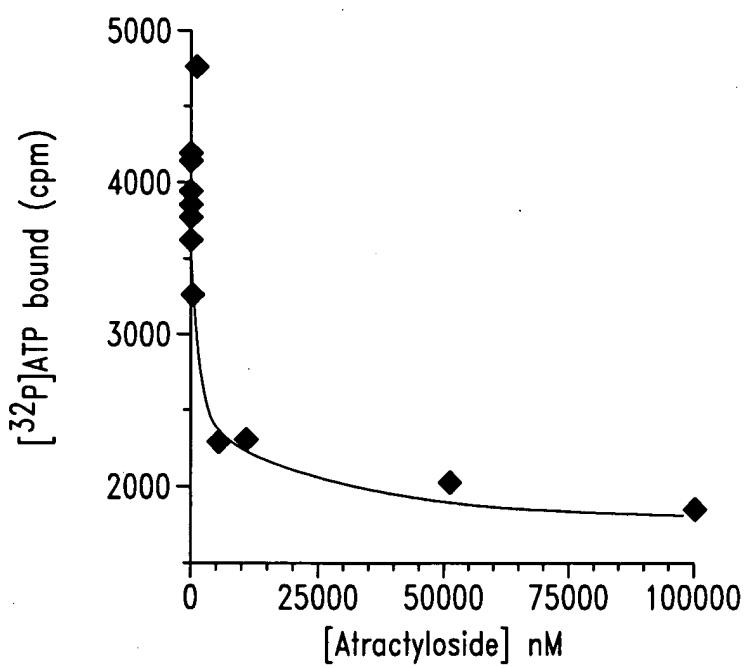


Fig. 8

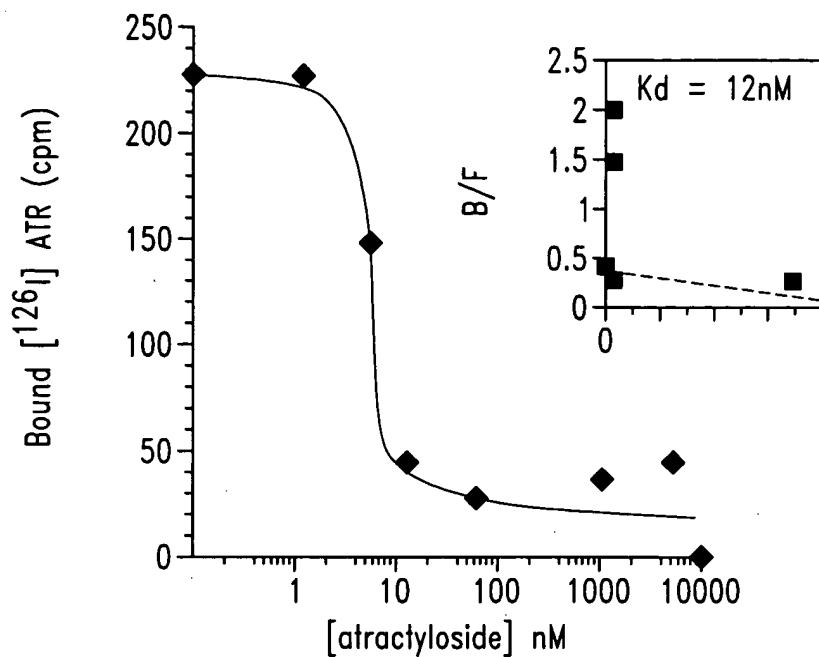


Fig. 9

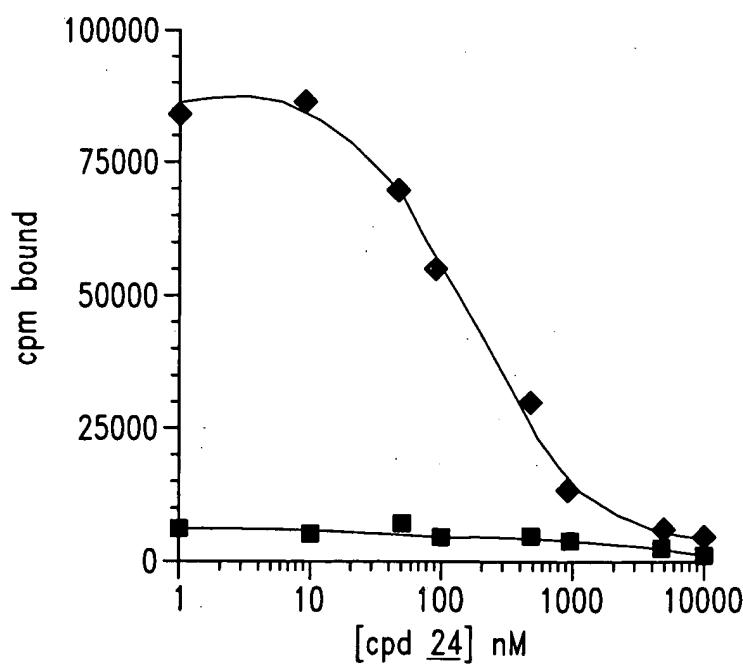


Fig. 11

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

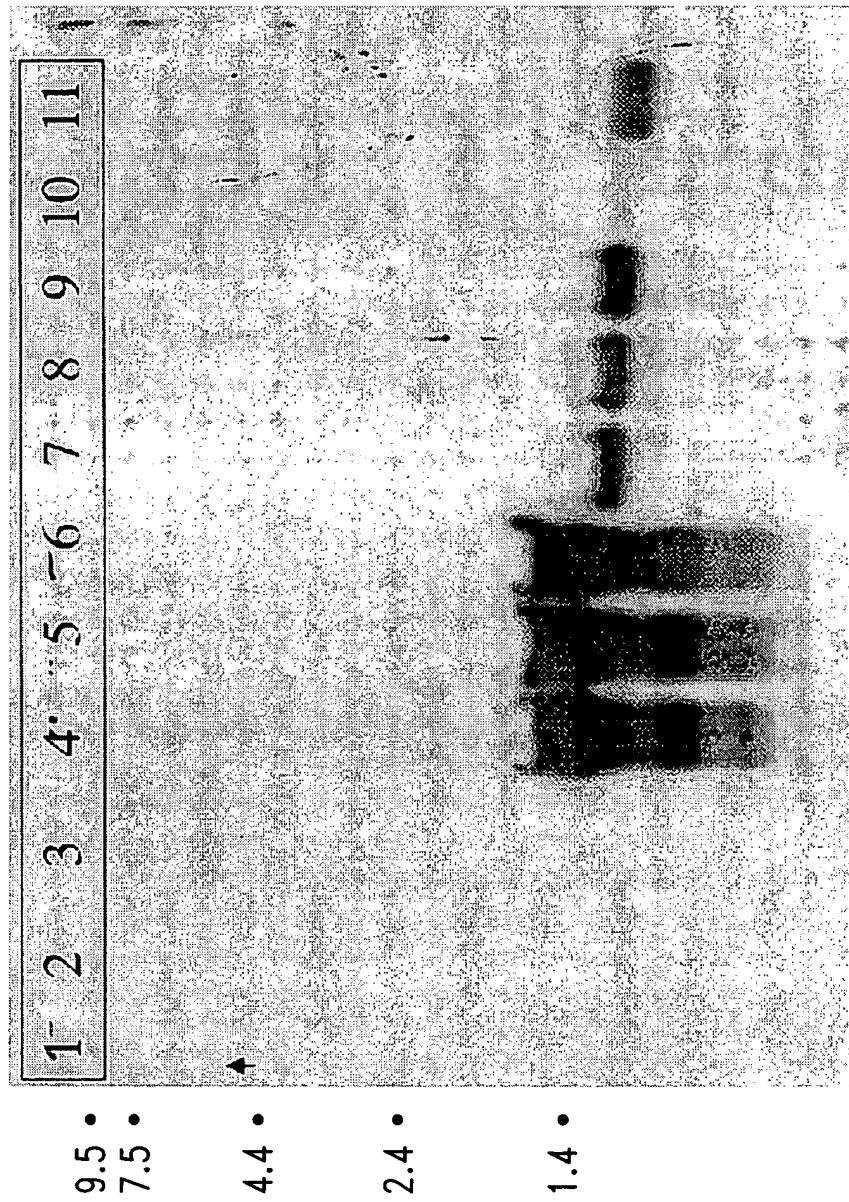


Fig. 10

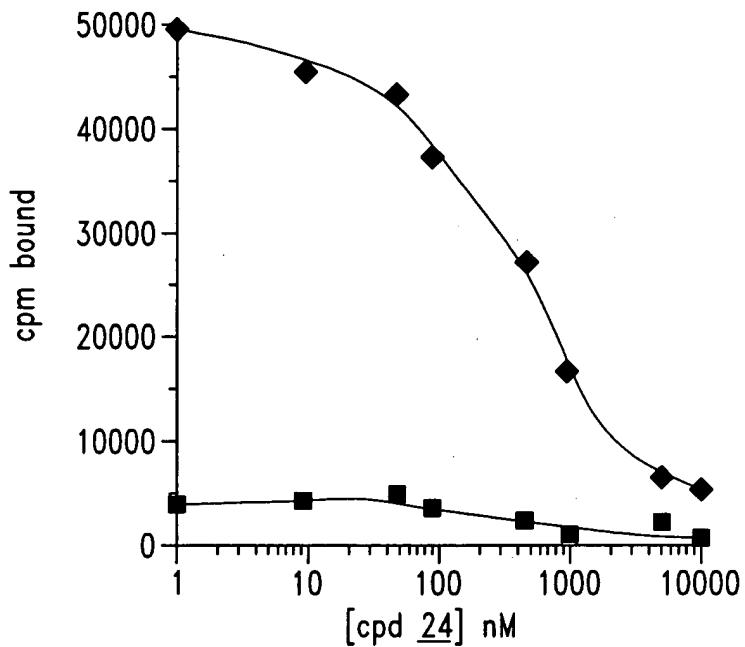


Fig. 12

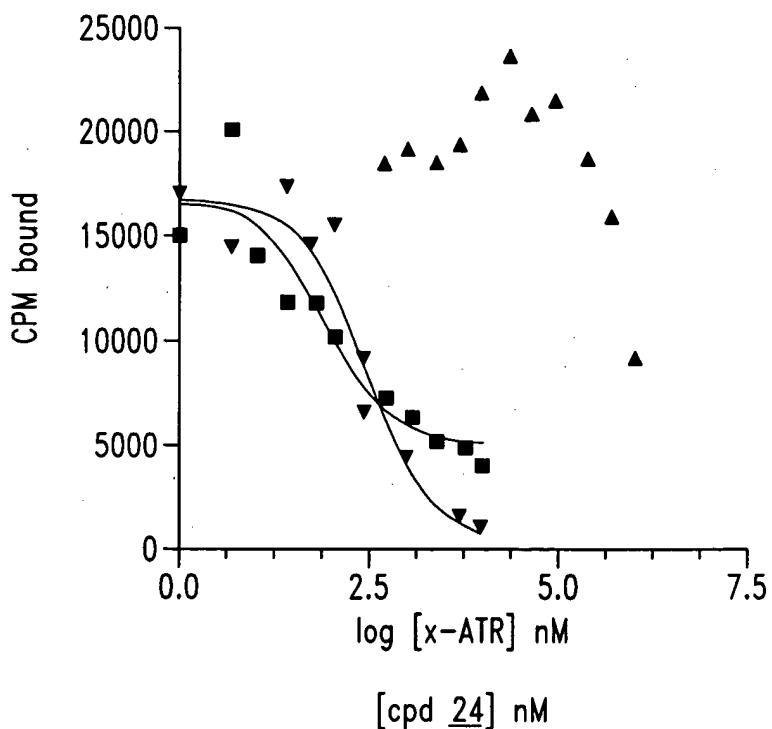


Fig. 13

Title: PRODUCTION OF ADENINE NUCLEOTIDE TRANSLOCATOR (ANT), NOVEL ANT LIGANDS AND
SCREENING ASSAYS THEREFOR "REPLACEMENT SHEET"

Inventor(s): Christen M. Anderson et al. Express Mail No. EV336618748US Docket No. 660088.420C1

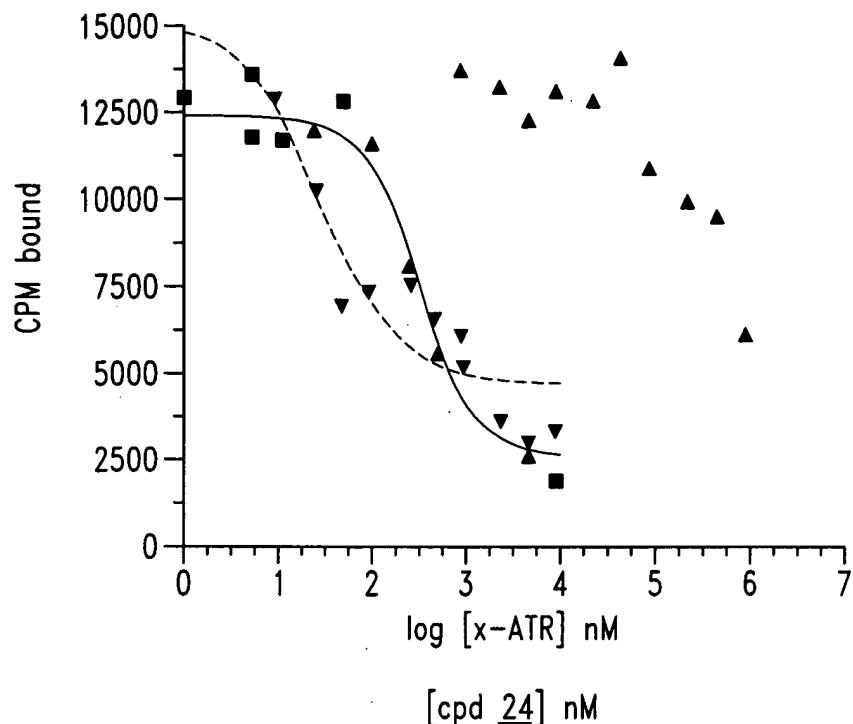


Fig. 14

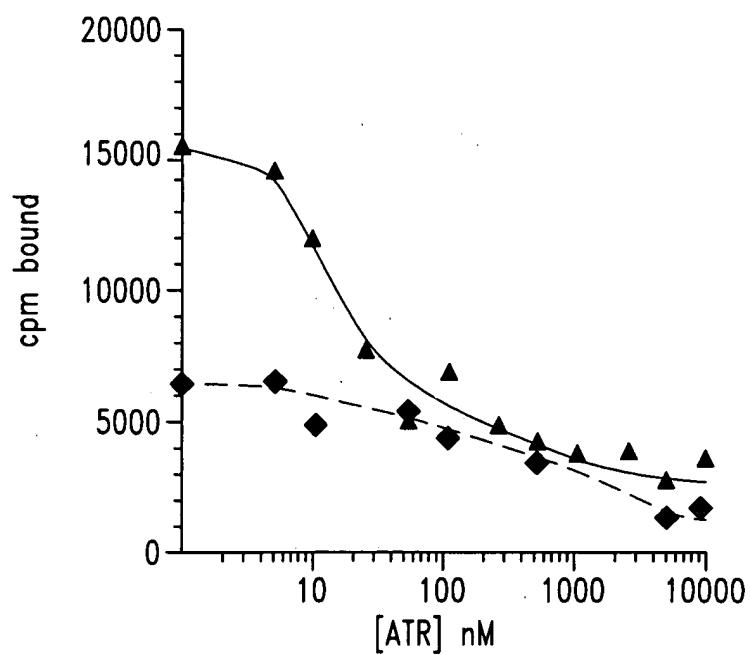


Fig. 15

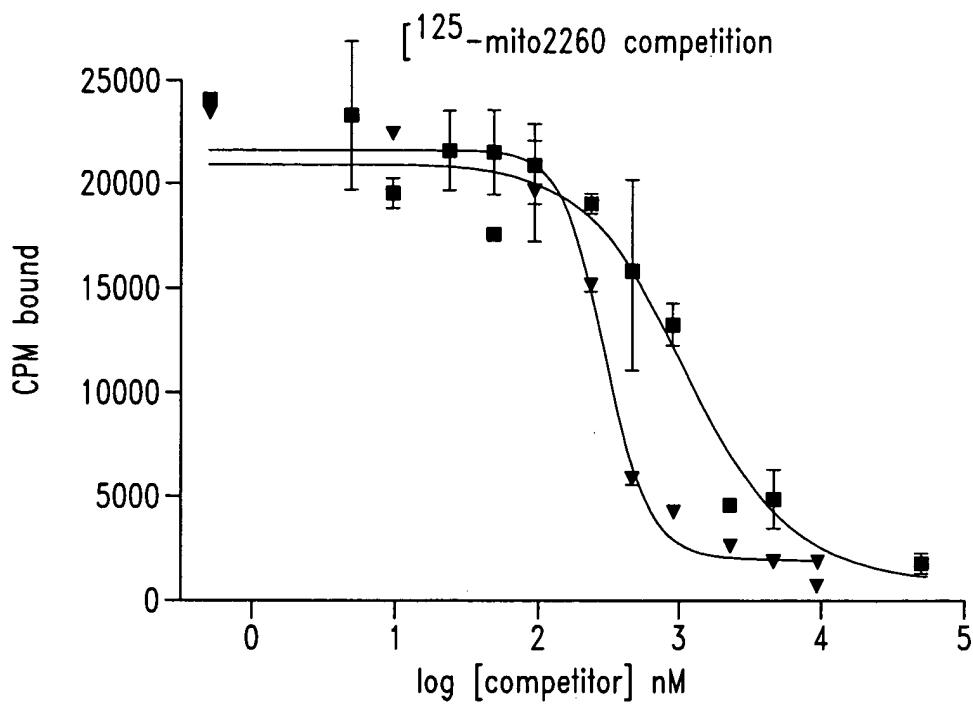


Fig. 16

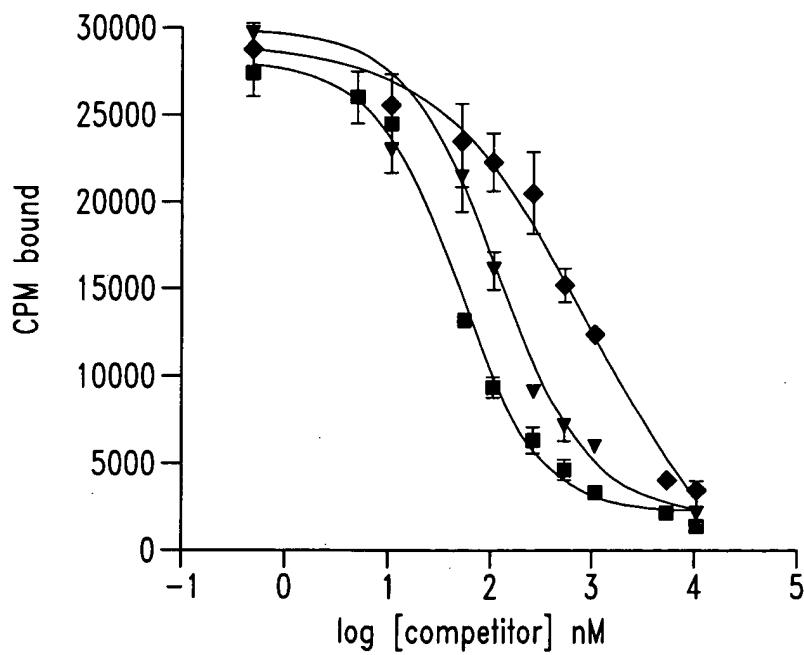


Fig. 17

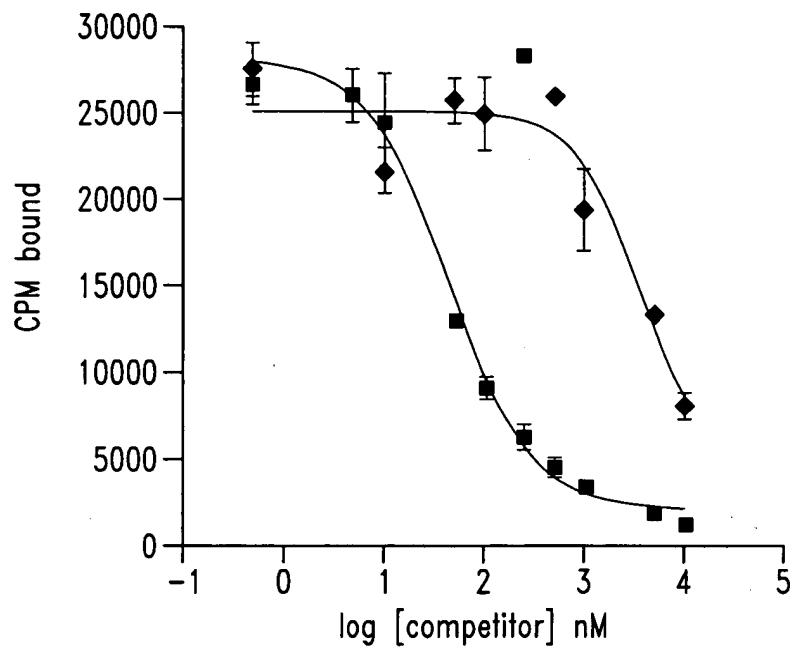


Fig. 18

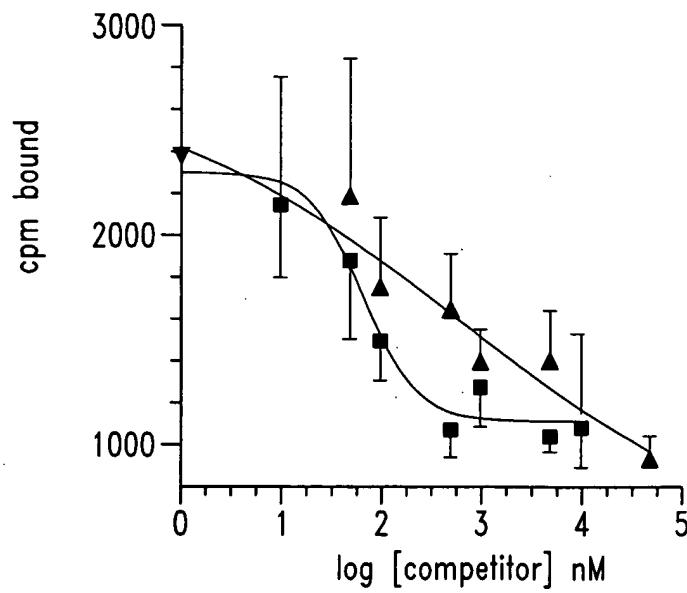


Fig. 19